Handbook of Residue Analytical Methods for Agrochemicals

VOLUME 1 and VOLUME 2

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DropBooks

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Preface

The agrochemical industry is, globally, one of the most heavily regulated industries today. Extensive product chemistry, environmental fate, residue chemistry, ecotoxicology, and mammalian toxicology data are required to support the registration and reregistration of all crop protection products. This information is used not only to conduct human dietary and worker exposure risk assessments but also to determine the potential impact of the agrochemicals and their degradation products/metabolites on the environment and sensitive ecosystems. The quality of the residue data, including the reliability and sensitivities of the analytical methods and the validity of the collected biological/environmental samples, is critical to the acceptability and validity of the risk characterization/assessment. Differences in testing guidelines between the various regulatory authorities and the lack of standardization in test method specifications further complicate the interpretation and broad application of the exposure data.

Significant progress has been achieved in residue analytical technology in the past 50 years. Today's residue analytical methodology detects multiple analytes routinely at the nanogram per kilogram (ppt) level in a wide variety of sample matrices with a high level of selectively and accuracy. The role of the residue analytical chemist is no longer limited to the development and validation of analytical methods but also includes design and conduct of complex field crop residue and environmental monitoring studies. This is a real challenge, especially when studies are conducted under the strict Good Laboratory Practices guidelines.

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Recognizing the diverse and rapid growth of residue chemistry as an important scientific discipline, Dr Terry Roberts, Founding Editor of the *Handbook of the Residue Analytical Methods of Agrochemicals*, organized this publication effort in 1999. The editorial team includes Dr Hiro Aizawa (Hiro Research Consultancy), Dr Al Barefoot (DuPont Crop Protection) and Dr John Murphy (Bayer CropScience). The scope/objective of this handbook is to present to the reader a comprehensive overview of current global regulatory requirements and the application of various analytical technologies (chromatographic and non-chromatographic) to residue analysis. Best practices to conduct various crop residue and field monitoring studies and detailed method procedures for the determination of major classes of agrochemicals, as well as individual compounds, are key components of this handbook.

This handbook consists of two volumes and approximately 80 individual chapters. The editorial team acknowledges the high quality of the contributions from the regulatory, academic, and industrial researchers around the world. It is their commitment in time and effort that make this a successful publication project. Each chapter was reviewed by at least one editor and often by other technical experts. The editorial team acknowledges the generous advice and reviews provided by our colleagues from DuPont Crop Protection (Dr Wynn John, Dr Chuck Powley) and Bayer CorpScience (Dr Lou Russo), the US EPA (Dr Alex Krynitsky) and the USDA ARS (Dr David Smith). We would also appreciate comments, feedback and upgrades from the readers, so that correction and improvement can be made for later editions or printings.

The editorial team is also grateful for the valuable support from the Publisher (John Wiley & Sons Ltd.), in particular Ms Lynette James, and from the Project Manager (Gray Publishing), in particular Ms Lesley Gray, for their efficient coordination during the planning, review and production phase of this publication effort.

Finally, this handbook is dedicated to all past and present residue analytical chemists. It is their vision and creativity that continues to push back the frontier of residue analytical technology.

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Newark, Delaware
December, 2002

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Introduction

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1 Introduction

The first generation of pest control agents, consisting largely of botanicals and inorganic substances containing copper, lead, sulfur, or arsenic, received little regulatory interest, and thus there was little monitoring of the products applied or of the residues remaining from them. Visual monitoring could be done for some materials, such as Paris Green, which was blue–green from its copper content, or lead arsenate, which often left a white residue on apples and other produce because of the high application rates. Simple gravimetric, titrimetric, or colorimetric methods could be used to quantify residues of many agents, including copper-, arsenic-, sulfur-, and lead-containing products or their derivatives.[1] Thoroughly washing treated fruit and fresh vegetables probably removed most residues since the materials in use were largely nonsystematic and water soluble or water wettable, lessening their hazard to the consumer.

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This situation changed significantly with the introduction of second-generation pest control agents, largely synthetic organics such as DDT, 2,4-D, and ethyl parathion, from the 1940s on. These chemicals had a number of qualities which invited heightened consumer concern, regulatory attention, and monitoring activity:

1. They were more widely used, some would argue overused, compared with first-generation products.
2. They were applied at such rates that their residues were not visible or detectable to the consumer.
3. Many were acutely toxic and/or chronically toxic to humans, domestic animals, and wildlife. Their ability to cause tumors, at least in laboratory animals, was of particular concern.
4. Their residues were mobile, systemically within plants and environmentally in air, water, soil, and food chains.
5. Many degraded/metabolized to products which had different toxicity and dissipation characteristics than the parents.

Regulation was developed in the 1950s and 1960s to include legal limits (tolerances) for residues on foods and in feeds and, with time, in water and air.[2] Enforcing these regulations required analytical methods of ever-increasing sophistication and

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sensitivity as public concern grew with each new residue-related crisis or toxicology finding. Early efforts at regulating residues confounded the situation. Legislators introduced a 'zero tolerance' concept for pesticides that produced cancer in experimental animals, for agricultural crops or food animal products such as milk and butter. However, as analytical methods improved, what was 'zero' by prior methods and instrumentation became detectable. Zweig[3] described three incidents in the 1950s and 1960s that showed the futility of zero tolerances.

One was the analytical finding, using a new method, of aminotriazole residues on cranberries from Oregon and Massachusetts in 1959, the week before Thanksgiving. The fungicide was a carcinogen with a 'zero tolerance'. The Food and Drug Administration (FDA) confiscated most of the cranberry harvest of 1959 and even some canned products from previous years. This gave a clear signal that 'zero' was a moving analytical target. Veteran residue chemists refer to the periods before and after 1959 as BC and AC – before and after cranberries!

A second was the 1960 finding of chlorinated insecticides, using a paper chromatography method perfected by Mills[4] and Mitchell,[5] and by the Schecter *et al.*[6] colorimetric method, in butter shipped from the mainland to Hawaii. This finding resulted in seizure of butter, milk, and other dairy products and posed a major dilemma for the government. Clearly, low residues of organochlorine insecticides in animal feed, which was practically unavoidable given the widespread use of these materials and their stability, could transfer to animals and dairy products in quantities detectable by residue analytical methods. The development of the electron capture detector for gas chromatography (GC) at about the same time[7] foreshadowed even more challenges for 'zero tolerance.'

The third was the publication, in 1962, of 'Silent Spring',[8] which revealed to a previously unaware public the extent of contamination of food with pesticide residues which were undetected by prior methods, and raised the possibility of irreversible harm to wildlife. The outcry which followed resulted in increased funds for research on better analytical methods for monitoring, as well as more extensive toxicology, environmental fate, and ecological effects studies. 'Silent Spring' set in motion a series of actions including the US Department of Health, Education, and Welfare Report ('Mrak Commission'),[9] establishment of the Environmental Protection Agency,[10] and the banning of dichlorodiphenyltrichloroethane (DDT) for agricultural uses in the USA in 1972, a century after it was discovered.[11]

With this backdrop, pesticide residue analysis grew and matured from, roughly, the 1950s to the present. Early advances and applications are published in such primary outlets as the *Journal of the Association of Official Analytical Chemists* (now *Journal of AOAC International*), *Journal of Agricultural and Food Chemistry*, *Analytical Chemistry*, and *The Analyst*. Secondary references or compendia include those by Gunther and Blinn,[1] Gunther,[12] Zweig,[13] and Moye[14] and the 'Pesticide Analytical Manual'.[15] The Association of Official Agricultural Chemists [later named the Association of Official Analytical Chemists (AOAC) and now AOAC International], the American Chemical Society's Division of Pesticide Chemistry (now the Agrochemical Division), and the International Union of Pure and Applied Chemistry (IUPAC) pesticide congresses were (and still are) popular meeting grounds for residue chemists.

A cadre of analytical agricultural chemists specializing in pesticide residue analysis emerged at a few North American, European and Japanese Universities, regulatory

agencies, food companies, and agricultural chemical companies. These chemists proved equal to the challenges posed by changing regulations, new toxicological findings, societal concerns, and the occasional crises. Colorimetry, polarography, and both paper and thin-layer chromatography provided minimum analyte detectabilities of 10^{-5}–10^{-8} g (10 μg–10 ng).[3] GC with element-selective detectors or electron capture detection (ECD) provided analyte detection limits of 10^{-9}–10^{-12} g (1 ng–1 pg). Hyphenated techniques, such as gas chromatography/mass spectrometry (GC/MS), gas chromatography/tandem mass spectrometry (GC/MS/MS) and high-performance liquid chromatography/mass spectrometry (HPLC/MS) also gave analyte detectabilities of 10^{-9}–10^{-12} g, but with exceptional, often single analyte selectivity. Enzyme-linked immunosorbent assay (ELISA) and other antibody-based immunoassays operate in the same range, often at significantly reduced costs.[16]

When pushed to the limit by overriding human health concerns, residue chemists have achieved detection limits of 1 ppt (1 ng kg^{-1}) or even into the low ppqr (1 pg kg^{-1}) range. An example at the 1 ppt level is provided by methods for 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in milk[17] and TCDD in adipose tissue.[18] For relatively clean matrices such as water and air, preconcentration on solid-phase adsorbents followed by GC or gas chromatography/mass spectrometry (GC/MS) can provide detection limits of 1 ng m^{-3} and less for air (examples in Majewski and Capel[19]) and 1 ng L^{-1} and less for water (examples in Larson *et al.*[20]). A summary of units of weight and concentration used to express residue data is given in Table 1.

The improvement in detection limits (and in accuracy and precision) can be ascribed to at least four advances in techniques and instrumentation:

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1. advent of commercial ultraviolet (UV) visible spectrophotometers, beginning with the Beckman DU spectrophotometer, and associated derivatization techniques to form UV-absorbing or colored derivatives;
2. development of chromatography, with its unsurpassed ability to resolve individual chemical species;
3. development of class- and chemical-specific spray reagents (paper and thin-layer) and electronic detectors for GC and high-performance liquid chromatography (HPLC), using element-selective and electron capture (GC), UV visible (HPLC), and mass spectrometry (both GC and HPLC).

Table 1 Units of weight and concentration commonly employed in pesticide residue chemistry

Units of weight		Units of concentration	
Unit	Gram equivalents	Unit	wt/wt equivalent[a]
1 microgram (1 μg)	10^{-6}	1 part per million (ppm)	1 mg kg^{-1}
1 nanogram (1 ng)	10^{-9}	1 part per billion (ppb)	1 μg kg^{-1}
1 picogram (1 pg)	10^{-12}	1 part per trillion (ppt)	1 ng kg^{-1}
1 femtogram (1 fg)	10^{-15}	1 part per quadrillion (ppqr)	1 pg kg^{-1}
1 attagram (1 ag)	10^{-18}		

[a] For water, the density of which is 1 kg L^{-1}, the same units are used.

These high-profile developments were accompanied by improvements in technology such as electronics, particularly the advent of transistors and integrated circuit boards, fiber optics, and computer interfaces.

2 Relationship of pesticide residue analysis, regulation, and risk assessment

Pesticide residue chemistry has responded to the challenges posed by new regulations and, in fact, underpins the ability to make tolerances, action limits, permissible levels, and acceptable daily intakes work. The dramatic lowering of permissible limits for pesticides in food, water, and air in the 1970s prompted dramatic decreases in limits of detection of analytical methods. The establishment and enforcement of tolerances for new chemicals, such as glyphosate,[14] which were difficult analytical challenges, required considerable innovations by residue chemists. Innovations occurred with single residue methods (SRMs) and multiresidue methods (MRMs). The latter allows the monitoring of a broad range of pesticides (and significant transformation products) in the same sample of foods, feeds, and environmental media. The FDA's 1987 MRMs, for example, included 316 pesticides for which tolerance levels had been set, 74 pesticides with temporary and pending tolerances, 56 pesticides with no Environmental Protection Agency (EPA) tolerance levels (i.e., those previously canceled or those used only in foreign countries), and 297 metabolites, impurities, inert ingredients, and other pesticide-associated chemicals.[21] These methods, or subsets of them, are used by the FDA for general commodity monitoring (ca 15 000 annual samples) and total diet study samples (234 food types sampled four times each year), again citing 1987 figures.[22] The MRMs of the FDA and other federal and state agencies have been summarized by Seiber.[23]

Other analytical challenges have been posed by new discoveries of toxic metabolites and formulation impurities. Included are ethylenethiourea (ETU) from the ethylenebis(dithiocarbamate) (EBDC) fungicides, dialkylnitrosamines from the dialkylamines used to formulate salts of phenoxy herbicides (as well as other sources), unsymmetrical dimethylhydrazine (UDMH) from daminozide (Alar) growth regulator, and aldicarb sulfoxide from aldicarb.[24] A challenge yet to be fully met by residue chemistry was posed by the most recent US pesticide-related law, the 1996 Food Quality Protection Act, which requires, among other things, that residue monitoring be conducted for foods significantly consumed by children and other subgroups, and that pesticide-related chemicals be screened and tested as endocrine-disrupting chemicals (EDCs).[25] The focus on EDCs has resulted in a world-wide effort to develop biological and chemical testing procedures for humans, wildlife, food, and environmental media.[26]

In addition to meeting the challenges posed by regulations, pesticide residue chemistry also plays a proactive role by detecting pesticides and their metabolites in environments where they were previously undetected and could pose undue hazards to people and/or wildlife. Examples include DDT and a host of other organohalogenated substances, now appropriately termed persistent organic pollutants (POPs), in a variety of samples ranging from human adipose tissue to bald eagles to Arctic

seals and polar bears.[27] The residue findings undoubtedly hastened the banning of DDT and other organochlorine insecticides in the USA and most industrial nations in the 1970s.

The advent of risk science and risk assessment has provided a framework for targeting the type of residue information that would be most useful to society.[28] Rather than relying on blind monitoring, i.e., without a hypothesis or framework, risk assessment emphasizes measuring exposures relevant to at-risk populations as a prelude to assessing impact, or potential impact, on the health of humans or wildlife. Exposure assessment requires good analytical chemistry to determine (or estimate) the average daily dose and the aggregate and cumulative exposure at several life stages. Without at least some quantitative exposure information, the uncertainty of assessing risks is too great to provide relevant, meaningful information. Exposure and risk assessment are, not surprisingly, cornerstones of the Food Quality Protection Act of 1996 (FQPA), indicating an even greater role for exposure analysis in the future.

3 Who does residue analysis and why

Ultimately, all food and environmental analyses are conducted to safeguard human health and the environment. Methods are selected and applied based upon specific needs within this broader framework, including adherence to regulations, tolerances, threshold levels, etc.[29]

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For companies that develop and register pesticides, the relevant laws (FIFRA and the FQPA) require the development of analytical methods which provide analytical data on the formulations used and measure the residues incurred during the testing phase leading to registration. These methods must be suitable for enforcement of tolerances and other restrictions after registration is granted. However, development of analytical methods is a tedious process. The methods need to account for the parent chemical as well as toxicologically significant formulation impurities, environmental breakdown products, and metabolites. Several iterations of method development may occur, because all of the impurities, metabolites, or breakdown products may not be known until each step of development of the new chemical is completed. Plant, animal, and soil metabolism studies and some studies of product breakdown by photolysis, hydrolysis, or microbial conversion are done using radiolabeled material. These studies are important for identifying conversion products, but the radioanalytical methods used are not applicable to monitoring the products and their residues after registration and use. The net result is that methods – often several for the same product – must be developed or provided by the registrant for monitoring food and feed, as well as soil, water, air, and nontarget organisms such as fish and other wildlife. Methods which have been thoroughly validated will be published in such compendia as the FDA Pesticide Analytical Manual[15] or the 'Official Methods of Analysis of the Association of Official Analytical Chemists'.[30]

In addition to regulatory agencies, the US Department of Agriculture (USDA), through its Cooperative State Research Education and Extension Service (CSREES) and the Agricultural Research Service (ARS), funds or carries out the development of analytical methods and the collection of residue data in studies for registration

of pesticides in 'minor use' situations through the IR-4 (Interregional Project No. 4) program. Minor crops are those crops, such as strawberries, apricots, broccoli, etc., whose acreage or usage of pesticides is too small to warrant the time and expense of the registrant alone to conduct tests needed to add the crop or use to the label. The use, however, may still be important to farmers who grow these crops. IR-4 residue research is carried out at one of the four IR-4 Leader Laboratories located at Cornell University, University of California, Davis, Michigan State University, and the University of Florida or at one of the satellite laboratories or field locations of the Leader laboratories. The ARS has a parallel network of laboratories and field sites to conduct IR-4 work.

The analytical methods required by agencies that conduct or oversee monitoring for pesticide residues may be different from those submitted by the registrant or developed by IR-4 laboratories or other groups. Monitoring agencies usually conduct multiresidue analyses, as noted above for the FDA, and thus may modify the submitted method or, more likely, incorporate the newly registered product in an existing multiresidue method published in the 'Pesticide Analytical Manual',[15] Vol. I (see also other discussions[21,31]). In addition to US organizations involved in monitoring pesticides in foods (Table 2), there are a number of international agencies and governmental organizations with expertise in pesticide residue analysis. These include the ISO (International Organization for Standardization), which includes 130 countries, AOACI (Association of Official Analytical Chemists International), IUPAC (International Union of Pure and Applied Chemistry), Codex Alimentarius, OECD (Organization for Economic Cooperation and Development), and FAO/WHO (Food and Agriculture Organization of the United Nations, World Health Organization). These organizations have initiatives to standardize methods and follow established protocols for producing acceptable data, and, in several cases, for carrying out monitoring activities.[32]

The collection of residue monitoring data, begun in the 1950s (and reported in the *Pesticide Monitoring Journal* as well as other outlets), has played a major role in understanding how residues are deposited and dissipated. Unfortunately, much of the older monitoring data is of limited utility, because the samples were not properly handled and preserved, the methods were not validated for precision and accuracy, and/or the results were not confirmed with an independent method; any of these deficiencies is enough to cast doubt on the quality of the data. Because analytical data are increasingly used for making regulatory or economic decisions that can affect the availability of chemicals, their safe handling, and the safety of the food supply, there has been much more emphasis, including regulatory requirements, that residue chemists pay close attention to the quality and meaning of the data they generate. Accreditation, quality assurance (QA)/quality control (QC) and Good Laboratory Practice (GLP) are integral components of a pesticide residue chemistry program, just as they are for toxicology laboratories.[33] Unfortunately, these new requirements with associated certification, chain-of-custody, record keeping, archival preservation and other requirements may increase the time and cost of residue procedures significantly. However, this extra effort is compensated for by the gain in confidence in the quality of the data and their comparability from one laboratory to another.

Table 2 Agencies and other organizations in the USA that conduct analyses for pesticide residues in foods [23]

Name	Purview
Federal	
Environmental Protection Agency	Reviews and checks out analytical methods for pesticides submitted by registrants
Food and Drug Administration	Monitors residues in imported and domestic food, including processed food
Food Safety and Inspection Service	Monitors residues in meat and poultry
Agricultural Marketing Service	Conducts market basket surveys
Fish and Wildlife Service	Monitors pesticides in fish and wildlife
State	
California Department of Food and Agriculture	Monitors pesticides and other contaminants in, primarily, fruits, and vegetables
Florida Department of Agriculture	Monitors pesticides and other contaminants in raw and processed foods
Texas, New York, Oregon, Washington, Massachusetts and other states	Monitor foodstuffs of specific interest to those states
Universities	
Cornell University, University of California, Davis, University of Florida, Michigan State University, and various satellite university laboratories	Conduct analyses for pesticides crops as part of the USDA IR-4 Minor Use registration program
Industry	
National Food Processors Association	Monitor pesticide residues, other additives/ contaminants in fresh and processed commodities
General Mills, Del Monte, Campbell, and other food companies	Monitor pesticides and other chemical contaminants for their company's products
Dow, DuPont, Syngenta, Bayer CropScience, Monsanto, and other chemical companies	Conduct analytical support for their own products in food and environmental media
Private Laboratories	
Commercial analytical laboratories	Conduct analyses for pesticides and other toxicants (metals, solvents, additives) in foods, soil, water, and wastes, under contract

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4 Challenges

Pesticide residue chemistry has developed largely by adapting techniques and instrumentation to the unique problems of ultra-low level analysis in complex matrices. New developments in molecular biology are providing new techniques, such as those of proteomics and genomics, which may lead to creating biologically based detection methods, further refinements of immunoassay and other antibody-based methods**,** and whole new classes of biosensors. Coupling the exciting advances in molecular biology with the already strong analytical chemistry underpinnings of pesticide residue analysis can benefit both areas. Applying the biosensor process to measuring residues where they count – in specific cells, and at specific receptors – may lead to a better understanding of the biological significance of residues. Related to this, crops that

are genetically modified to incorporate pest control agents pose new challenges for residue chemists in detecting the genetically modified material through the distribution chain to the consumer's diet and to nontarget species – further areas for applying tools of molecular biology in residue analysis.[34]

Residue chemists will need to continue to improve the speed of analysis. In situ measuring methods that can be applied in the field or processing plant or retail outlet would be particularly useful, so that decisions can be made rapidly which might avert toxicity to humans or wildlife, potential residue problems or unnecessary economic loss.

In addition, further automation will be needed in what is still very much a hands-on art. Autoinjectors coupled to complete analytical data systems and readers for 96-well plates are the beginning of what will continue to be a necessary trend of residue chemistry. The application of the techniques of combinatorial chemistry/biochemistry, which has produced screening methodology for handling many variables, might be appropriate to residue chemistry.

The following pages of this book will show how far pesticide residue chemistry has come and provide a platform for the many advances still in the offing.

References

1. F.A. Gunther and R.C. Blinn, 'Analysis of Insecticides and Acaricides,' Interscience, New York (1955).
2. G.J. Marco, R.M. Hollingworth, and J.R. Plimmer (eds), 'Regulation of Agrochemicals. A Driving Force in their Evolution,' American Chemical Society, Washington, DC (1991).
3. G. Zweig, 'The vanishing zero: the evolution of pesticide analysis,' in "Essays in Toxicology," ed. F.R. Blood, Academic Press, New York, Vol. 2 (1970).
4. P.A. Mills, *J. Assoc. Off. Agric. Chem.,* **42**, 734 (1959).
5. L.C. Mitchell, *J. Assoc. Off. Agric. Chem.,* **41**, 781 (1958).
6. M.S. Schechter, S.B. Soloway, R.A. Hayes, and H.L. Haller, *Ind. Eng. Chem. Anal. Ed.,* **17**, 704 (1945).
7. K.P. Dimick and H. Hartman, *Residue Rev.,* **4**, 150 (1963).
8. R. Carson, 'Silent Spring,' Houghton, Boston, MA (1962).
9. US Department of Health, Education, and Welfare, 'Report of the Secretary's Commission on Pesticides and their Relationship to Environmental Health. Parts I and II,' US Government Printing Office, Washington, DC (1969).
10. 'Code of Federal Regulations,' Title 3, 1966–1970 Comp. (1970).
11. R.L. Metcalf, *J. Agric. Food Chem.,* **21**, 511 (1973).
12. F.A. Gunther, (ed.), 'Residue Reviews (Residues of Pesticides and Other Foreign Chemicals in Foods and Feed),' Academic Press, New York, and Springer, Berlin (1962) (subsequent volumes are up to Vol. 171, 2001, edited by G. Ware).
13. G. Zweig (ed.), 'Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives,' Academic Press, New York, Vol. 1 (1963) (subsequent volumes are up to Vol. 17, 1989, edited by J. Sherma).
14. H.A. Moye, 'Analysis of Pesticide Residues,' Wiley, New York (1981).
15. Food and Drug Administration, 'Pesticide Analytical Manual,' US Department of Health and Human Services, Washington, DC (1994).
16. H.A. Moye, 'Enzyme-linked immunosorbent assay (ELISA),' in 'Pesticide Residues in Foods: Methods, Techniques, and Regulations,' W.G. Fong, H.A. Moye, J.N. Seiber, and J.P. Toth (eds), Wiley, New York, Chapt. 6 (1999).
17. M.L. Langhorst and L.A. Shadoff, *Anal. Chem.,* **52**, 2037 (1980).

18. D.G. Patterson, J.S. Holler, C.R. Lapeza, Jr, L.A. Alexander, D.F. Groce, R.C. O'Connor, S.J. Smith, J.A. Liddle, and L.L. Needham, *Anal. Chem.,* **58**, 705 (1986).
19. M.S. Majewski and P.D. Capel, 'Pesticides in the Atmosphere: Distribution, Trends, and Governing Factors,' Ann Arbor Press, Chelsea, MI, Chapt. 2 (1995).
20. S.J. Larson, P.D. Capel, and M.S. Majewski, 'Pesticides in Surface Waters: Distribution, Trends, and Governing Factors,' Ann Arbor Press, Chelsea, MI, Chapt. 2 (1997).
21. Office of Technology Assessment (OTA), 'Pesticide Residues in Food: Technologies for Detection,' US Congress, Office of Technology Assessment, Washington, DC (1988).
22. B.M. McMahon and J.A. Burke, *J. Assoc. Off. Anal. Chem.,* **70**, 1072 (1987).
23. J.N. Seiber, 'Extraction, cleanup, and fractionation methods,' in "Pesticide Residues in Foods," ed. W.G. Fong, H.A. Moye, J.N. Seiber, and J. P. Toth, Wiley, New York, Chapt. 2 (1999).
24. J.N. Seiber, 'Analytical chemistry and pesticide regulation,' in "Regulation of Agrochemicals. A Driving Force in their Evolution," ed. G.J. Marco, R.M. Hollingworth, and J.R. Plimmer, American Chemical Society, Washington, DC, Chapt. 10 (1991).
25. S.L. Johnson and J.E. Bailey, 'Food Quality Protection Act of 1996,' in "Pesticides: Managing Risks and Optimizing Benefits," N.N. Ragsdale and J. N. Seiber (eds), ACS Symposium Series 734, American Chemical Society, Washington, DC, pp. 8–15 (1999).
26. L.H. Keith, T.L. Jones-Lepp, and L.L. Needham, 'Analysis of Environmental Endocrine Disruptors,' ACS Symposium Series 747, American Chemical Society, Washington, DC (2000).
27. C. Bernes, 'Persistent Organic Pollutants. A Swedish View of an International Problem,' Swedish Environmental Protection Agency, Stockholm (1998).
28. National Research Council, 'Pesticides in the Diets of Infants and Children,' National Academy Press, Washington, DC (1993).
29. J.N. Seiber, 'Analysis of chemical toxicants and contaminants in foods, in "Food Toxicology," ed. W. Helferich and C. K. Winter, CRC Press, Boca Raton, FL, Chapt. 9 (2001).
30. Association of Official Analytical Chemists, 'Official Methods of Analysis of the Association of Official Analytical Chemists,' 15th edition, AOAC, Arlington, VA (1990).
31. W.G. Fong, H.A. Moye, J.N. Seiber, and J.P. Toth, "Pesticide Residues in Foods: Methods, Techniques, and Regulations," Wiley, New York (1999).
32. A. Ambrus, 'Quality of residue data,' in "Pesticide Chemistry and Bioscience: The Food–Environment Challenge," ed. G.T. Brooks and T.R. Robers, Royal Society of Chemistry, Cambridge, pp. 339–350 (1999).
33. W.G. Fong, 'Regulatory aspects: pesticide registration, risk assessment and tolerance, residue analysis, and monitoring,' in "Pesticide Residues in Foods: Methods, Techniques, and Regulations," ed. W.G. Fong, H.A. Moye, J.N. Seiber, and J.P. Toth, Wiley, New York, Chapt. 7 (1999).
34. National Research Council, 'The Future Role of Pesticides in U.S. Agriculture,' National Academy Press, Washington, DC (2000).

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Abbreviations and acronyms

A	Adenine
Ab	Antibody
ACCD	1-Aminocyclopropane-1-Carboxylic acid deaminase
ACCS	Aminocyclopropane carboxylic acid synthase
Ag	Antigen
ALS	Acetolactate synthase
ASE	Accelerated solvent extraction
AV	Application verification
bDNA	Branched DNA
bp	Base pairs
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
C	Cytosine
CaMV	Cauliflower mosaic virus
CCD	Charge-coupled device
CD	Compact disk
CFR	Code of Federal Regulations
CMC	1-Cyclohexyl-3-(2-Morpholinoethyl)carbodiimide metho-*p*-Toluenesulfonate (same as Morpho CDI)
CMV	Cucumber mosaic virus
C_T	Threshold cycle
DAM	DNA adenine methylase
DCC	Dicyclohexylcarbodiimide
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ECD	Electron capture detection
EDC	1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide HCl
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EPSPS	5-Enolpyruvylshikimate-3-Phosphate synthase
EU	European Union
FATUS	Foreign Agricultural Trade of the US
FDA	Food and Drug Administration
FIIA	Flow injection immunoassay
FQPA	Food Quality Protection Act
FRET	Forster resonance energy transfer
G	Guanine
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
GE	Genetically engineered
GLC	Gas–liquid chromatography
GLP	Good Laboratory Practice
GM	Genetically modified
GMO	Genetically modified organism
GOX	Glyphosate oxidoreductase
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSA	Human serum albumin
I_{50}	The concentration of analyte that inhibits the immunoassay by 50%
IA	Immunoassay
IAC	Immunoaffinity chromatography
IgG	Immunoglobulin G
K_A	Equilibrium binding constant for the binding of analyte to antibody
K_D, K_{OC}	Soil sorption coefficients
K_H	Equilibrium binding constant for the binding of hapten to antibody
KLH	Keyhole limpet hemocyanin
λ_{max}	Wavelength of maximum absorption
LACPA	Latin American Crop Protection Association
LC	Liquid chromatography

LC/MS	Liquid chromatography/mass spectrometry
LLD	Lower limit of detection
LOD	Limit of detection
LOQ	Limit of quantitation
LPH	Horseshoe crab hemocyanin
LSC	Liquid scintillation counting
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectrometry
MBS	*m*-Maleimidobenzoyl-*N*-Hydroxysuccinimide
Morpho CDI	1-Cyclohexyl-3-(2-Morpholino-ethyl)carbodiimide metho-*p*-Toluenesulfonate (same as CDI)
MRL	Maximum residue limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSDS	Material safety data sheet
NAFTA	North American Free Trade Act
NHS	*N*-Hydroxysuccinimide
NPD	Nitrogen–phosphorus detection
NPTII	Neomycin phosphotransferase II
OD	Optical density
OPPQ	Office of Plant Protection and Quarantine
OPPTS	Office of Prevention, Protection and Toxic Substances
PAT	Phosphinothricin acetyltransferase
PBA	Phenoxybenzoic acid
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PG	Polygalacturonase
pK_a	Acid dissociation constant
PPQ	Plant protection and quarantine
PRSV	Papaya ringspot virus
PVP	Polyvinylpyrrolidine
QA	Quality assurance
QC	Quality control
r	Regression correlation coefficient
R^2	Regression coefficient of determination
RCA	Rolling circle amplification
S_w	Water solubility
SDS	Sodium dodecyl sulfate
SFE	Supercritical fluid extraction
SOP	Standard operating procedure
SPE	Solid-phase extraction
SPR	Surface plasmon resonance
T	Thymine
T_a	Annealing temperature
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin
T_m	Melting temperature
TDR	Time domain reflectometry
Ti	Tumor-inducing
TOF	Time-of-flight
TPS	Template preparation solution
USDA	United States Department of Agriculture
USDA GIPSA	United States Department of Agriculture Grain Inspection Protection Service
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
UV/VIS	Ultraviolet/visible
WMV2	Watermelon mosaic virus2
ZYMV	Zucchini yellow mosaic virus

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Regulatory guidance and scientific consideration for residue analytical method development and validation

Assessment of residue analytical methods for crops, food, feed, and environmental samples: the approach of the European Union

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1 Introduction

Plant protection products are widely used throughout the world to reduce the loss in crop production caused by harmful organisms and weeds. However, their usage poses potential risks to humans, animals and the environment, especially if used without having been evaluated for safety and without having been authorized. In order to minimize the risks and to facilitate the trade of plant protection products and agricultural produces within the common market, the European Community (EC) has adopted Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market.[1] As a result, the evaluation of the safety of active ingredients (a.i.) contained in plant protection products is now carried out on the basis of data requirements which are harmonized throughout the EC. For reasons of preventive health protection and protection of the environment, the use of plant protection products has to be limited to the minimum level compatible with effective crop protection. Maximum residue limits (MRLs) are established for crops and food. Member States are responsible for monitoring the compliance of foodstuffs with these MRL levels on a regular basis to ensure that no misuse of products has taken place. In view of the importance of the quality of water intended for human consumption, a general limit for crop protection products and toxicologically relevant metabolites/degradation products is also established for drinking water. For surface water, soil, and air, there are no harmonized limits; however, pesticide residue levels in these environmental compartments are regulated at the national level.

Residue analytical methods are needed to enforce these legally based limits or guidance values and to perform monitoring projects. For existing a.i., validated analytical procedures for only a few selected compounds have been published in journals or

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handbooks. But for many compounds in use and especially for new a.i., there are no sufficiently validated residue analytical methods available in open literature. Therefore, legal provisions are created to supply laboratories involved in post-registration control and monitoring with residue analytical methods for plant protection products. Analytical methods are required, as part of the registration data package, to be evaluated at national and/or at Community level.

The purpose of this article is to clarify the assessment of residue analytical methods in the context of Directive 91/414/EEC. After discussing the legal and historical background, requirements for enforcement methods as well as data generation methods are reviewed. Finally, an outlook over further developments in the assessment and validation of analytical methods is provided.

2 Legal background

2.1 General

Since the foundation of the European Communities was laid in 1952 with the European Coal and Steel Community (ECSC), the importance of the European Communities within their own borders and for the global economic system has increased. Starting with six European countries in 1952, the EC now comprises of 15 Member States, and enlargement negotiations are in progress. The European Communities have continued to develop, becoming the European Union (EU), an umbrella for the three extant European Communities, ECSC, European Atomic Energy Community (EURATOM), and European Community [EC, formerly European Economic Community (EEC)]. Institutions involved in the legislative process are the Council of the European Union, usually known as the Council of Ministers (of the Member States), the European Commission (the administration of the EC) and, with limited powers, the European Parliament. The Court of Justice ensures that the law is observed in all Community and Member State activities. Community law may take the following forms: regulations are applied directly in all Member States without the need for national measures to implement them.[2] Directives bind Member States to achieve the objectives while leaving the national authorities the power to choose the form and the means for implementing the Directives. Decisions are binding in all their aspects for those to whom they are addressed.[2] A decision may be addressed to any or all Member States, to undertakings or to individuals. Recommendations are not legally binding. Community legislation is published in the *Official Journal of the European Communities* in all official languages of the EC. Guidance documents do not intend to produce legally binding effects and by their nature do not prejudice any measure taken by a Member State within its implementation of Directives. Details of the legal background are described, for example, by Wirsing *et al.*[2]

2.2 Council Directive 91/414/EEC

Until 1991, all Member States of the EC applied their own registration regime for plant protection products and operated independently with very little collaboration

between the countries in most cases. These individual regimes were considered to constitute a barrier to trade in plant protection products and agricultural produce within the internal market of the EC.

In order to set up a harmonized framework for the regulation of plant protection products in the EC, Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market was adopted and implemented in all Member States. Six annexes were established within this Directive, providing the basis for the harmonization of registration procedures and regulatory decisions (Table 1).

Through the adoption of Directive 91/414/EEC, a decision-making regime for determining the acceptability of a.i., which are denoted as active substances (a.s.) in the EU's legislation, was established. Authorization of plant protection products was still to be undertaken at national level by the individual Member States. A national authorization may be granted providing that the a.i. has been included in the 'positive Community list' of a.i. (Annex I to the Directive), and the 'uniform principles' for evaluation are applied, as defined in Annex VI to the Directive. Annex I inclusion of an a.i. is the result of a harmonized evaluation and decision-making procedure, performed on the basis of harmonized data requirements, as detailed in Annexes II and III to the Directive.

These annexes set out the requirements for the dossier to be submitted by applicants either for inclusion of an a.i. in Annex I or for authorization of a plant protection product. Active ingredients are listed in Annex I if their use and their residues, resulting from applications consistent with good plant protection practice [or Good Agricultural Practice (GAP)] do not have harmful effects on human and animal health, or on ground water or any unacceptable influence on the environment (Article 5 of the Directive). In order to take account of developments in science and technology, the inclusion of an a.i. in Annex I is limited to a period not exceeding 10 years to ensure that the inclusion is regularly reviewed to meet modern safety standards. Furthermore, Annex I listing is the prerequisite for the mutual recognition of authorizations between Member States, whereby one Member State is obliged to accept the evaluation and authorization prepared by another Member State in situations where the agricultural, plant health, and environmental (including climatic) conditions relevant to the use of the plant protection product are comparable in the regions concerned (Article 10 of the Directive).[2]

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2.3 Legislation related to MRLs

Pesticide residue levels in foodstuffs are generally regulated in order to:

- minimize the exposure of consumers to the harmful or unnecessary intake of pesticides
- allow control over the use of plant protection products
- permit the free circulation of products treated with pesticides as long as they comply with the established MRL.

The MRL for pesticide residues is the maximum concentration of a pesticide residue (expressed milligrams per kilogram) legally permitted in or on food commodities and

Table 1 Annexes of Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market and its implementation (status: published up to February 2002)

Annex	Content	Implementation	
Annex I	Active substances (a.s.)[a] authorized for incorporation in plant protection products	New as[b]	Existing as[c]
		Acibenzolar-S-methyl	Amitrol
		Azimsulfuron	Bentazon
		Azoxystrobin	λ-Cyhalothrin
		Cyclanilide	2,4-D
		Fenhexamid	Diquat
		Flupyrsulfuron-methyl	Fluroxypyr
		Iron(III) phosphate	Esfenvalerat
		Kresoxim-methyl	Glyphosate
		Paecilomyces	Imazail
		Prohexadion-calcium	Isoproturon
		Pymetrozine	Metsulfuron-methyl
		Pyraflufen-ethyl	Pyridat
		Spiroxamine	Thiabendazole
			Triasulfuron
			Thifensulfuron-methyl
Annex II	Requirements for the dossier to be submitted for the inclusion of an active substance in Annex I	Part A: Chemicals as	Directive
		Efficacy	93/71/EEC
		Physical-chemical properties	94/37/EC
	Part A: Chemical substances	Analytical methods	96/46/EC
	Part B: Microorganisms and viruses	Toxicology and metabolism	94/79/EC
		Residues	96/86/EC
Annex III	Requirements of the dossier to be submitted for the authorization of a plant protection product	Fate and behavior in the environment	95/36/EC
		Ecotoxicology	96/12/EC
	Part A: Chemical preparations	Part B: Microorganisms and viruses	Directive
	Part B: Preparations of microorganisms or viruses		93/71/EEC
			2001/36/EC
Annex IV	Risk phrases	In preparation	
Annex V	Safety phrases	In preparation	
Annex VI	Uniform principles for the evaluation of plant protection products	Directive 97/57/EC	

[a] Term for a.i. used in EU legislation.
[b] New a.s. are active substances not on the market of EC in protection products before 25 July 1993.
[c] Noninclusion has been decided for the following as after evaluation: azinphos-ethyl, chlozolinate, chlorfenapyr, cyhalothrin, dinoterb, DNOC, fentin-acetate, fentin-hydroxide, fenvalerate, ferbam, lindane, monolinuron, parathion, permethrin, propham, pyrazophos, quintozen, tecnazen, zineb.

animal feed. MRLs are based on GAP. These should reflect minimum quantities of pesticide necessary to achieve adequate pest control, applied in such a manner that the residues are as low as practicable. MRLs are also established at or about the limit of determination where there are no approved uses or where no residues occur when the pesticide is used according to GAP. MRLs are not toxicological limits but must be toxicologically acceptable. Exceeding the MRL is a violation of GAP.

Legislation at Community level dates back to November 1976 when Council Directive 76/895/EEC[3] established MRLs for 43 active substances in fruits and vegetables. These MRLs were based on the best data available at that time. These MRLs are gradually being reviewed and, where appropriate, replaced with MRLs based on more current information and higher standards.

Current pesticide MRL legislation is derived from/based on four Council Directives:

- Council Directive 76/895/EEC[3] establishing MRLs for fruits and vegetables
- Council Directive 86/362/EEC [4]establishing MRLs for cereals and cereal products
- Council Directive 86/363/EEC[5] establishing MRLs for products of animal origin
- Council Directive 90/642/EEC[6] establishing MRLs for products of plant origin, including fruits and vegetables.

Legislation for pesticide residues, including the setting of MRLs in food commodities, is a shared responsibility between the Commission and the Member States. To date, Community MRLs have been established for about 130 pesticide a.i. For pesticides and commodities where no Community MRL exists, the situation is not harmonized and the Member States may set MRLs at national levels to protect the health of its citizens.

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Where nonharmonized national MRLs exist, there is always a possibility of trade disputes. Until 1997, MRLs were established on raw commodities only. Directive 97/41/EC changed three important aspects of the work:

- it provided a mechanism to set MRLs in processed products and composite foodstuffs, based on the MRLs fixed for raw agricultural products
- it established a conciliation procedure through which cases where national MRLs led to barriers of trade within the Community could be resolved
- it transferred the competence for setting MRLs from the Council of the Member States to the Commission in Brussels.

Member States monitor the compliance of foodstuffs with these MRLs regularly. Inspections and monitoring should be carried out in accordance with the provisions of Council Directive 89/397/EEC[7] on the official control of foodstuffs, and Council Directive 93/99/EC[8] on additional measures concerning the official control of foodstuffs.

The MRLs are derived from data from supervised residue trials that are generally carried out in the context of food production. Specific conditions of feed production are not considered. Therefore, many practical problems for the official control of feed must be solved in future, e.g., application of transfer factors and the calculation of MRLs for mixed feed.

Besides national monitoring programs, the participation of each Member State in an EU-coordinated monitoring program is recommended. These monitoring programs

have existed since 1996, and are intended to provide an accurate dietary pesticide exposure throughout the EU and Norway. They will have covered all major pesticide–commodity combinations by the end of 2003. The choice of commodities includes the major components of the Standard European Diet of the World Health Organization.

In recent years, new legislation (Council Directive 99/39/EC) has placed severe restrictions on the use of pesticides in the production of food for infants and young children.

2.4 Legislation related to residues limits for soil, water, and air

The natural and socio-economic differences within the EU require the most decisions on the monitoring and enforcement of residues in the environment as well as measures to redress failures at local, regional, and national levels. Therefore, no harmonized limits for pesticides in soil and in air exist.

Because of the great importance of drinking water for human health, quality standards for pesticides in water were developed at Community level based on the precautionary principle.[9] Toxicological considerations were not taken into account to derive the general limit for pesticides.

Within the EU, many water-related Directives have been established over the past years. The most important one for the assessment of analytical methods for plant protection products is Directive 98/83/EC on the quality of water intended for human consumption.[10] According to Annex I Part B to the Directive, a general limit of 0.1 $\mu g\,L^{-1}$ applies uniformly to each individual pesticide. The sum of all individual pesticides detected may not exceed 0.5 $\mu g\,L^{-1}$. Only those pesticides which are likely to be present in a water supply need to be monitored. As a result, analytical methods used for water monitoring purposes must be able to determine pesticide residues at the 0.1 $\mu g\,L^{-1}$ level. As a contrast to the concept of setting MRLs, the concept of a general limit excludes specific considerations on the properties of individual a.i., e.g., toxicity. From an analytical point of view, this concept leads in some cases to inconsistencies regarding naturally occurring insecticides listed by the Commission such as carbon dioxide, rape seed oil, nitrogen, or naturally occurring herbicides like such as iron (II) sulfate and iron (III) sulfate. Moreover, additional specific limits apply to copper compounds (copper: 3 $mg\,L^{-1}$) and cyanide (50 $\mu g\,L^{-1}$).

For surface water, there are no legally binding limits except for parathion, HCH, and dieldrin in surface water intended for drinking water preparation (Directive 75/440/EEC). Possibly the establishment of the Water Frame Directive of 22 December 2000 will lead to harmonized quality standards for selected pesticides in surface water. Currently, provisions of Annex VI to Directive 91/414/EEC concerning the acceptable exposure of aquatic nontarget organisms are the basis for calculating guidance limits for assessing analytical methods for surface water.

2.5 Provisions for residue analytical methods

The first step to define data requirements and criteria for decision making for residue analytical methods was attempted in Council Directive 94/43/EC, establishing

Annex VI to Directive 91/414/EEC concerning the placing of plant protection products on the market. The section concerning residue analytical methods was not fully finalized when the Directive was first adopted. There were no provisions for methods to determine residues from a.i. and relevant metabolites in soil, water, and air. The criteria for foodstuffs partly proved to be not helpful for the practice of assessment (e.g., with regard to reproducibility, ISO 5725 requires validation in at least eight independent laboratories).

Although Directive 94/43/EC was later substituted by Council Directive 97/57/EC of 22 September 1997,[11] the provisions for analytical methods remained unchanged.

Commission Directive 96/46/EC of 16 July 1996, amending Annex II to the Directive 91/414/EEC, is the basis for the assessment of residue analytical methods for crops, food, feed, and environmental samples.[12] Provisions of this Directive cover methods required for post-registration control and monitoring purposes but not data generation methods. Because it is necessary to provide applicants as precisely as possible with details on the required information, the guidance document SANCO/825/00 rev. 6 dated 20 June 2000 (formerly 8064/VI/97 rev. 4, dated 5 December 1998)[13] was elaborated by the Commission Services in cooperation with the Member States. Moreover, this document provides guidance to Member States on the interpretation of the provisions of Directive 96/46/EC concerning minimum validation requirements for residue analytical methods.

For analytical methods used for generating data required in the field of residue behavior, environmental fate, and other fields, the guidance document SANCO/3029/99 rev. 4 was developed.[14]

According to guidance document 7109/VI/94 rev. 6, the development and validation of an analytical method for monitoring purposes and post-registration control are not subject to Good Laboratory Practice (GLP) regulation. However, where the method is used to generate data for registration purposes, for example residue data, these studies must be conducted according to GLP.[15]

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Table 2 Relevant legal provisions for residue analysis

Document	Year of publication	Scope
Directive 85/591/EEC	1985	Analytical methods for food control
Directive 89/397/EEC	1989	General principles of food control
Directive 94/43/EC (Annex VI of 91/414/EEC)	1994	Uniform principles for national authorizations
Directive 96/46/EC	1996	Data requirements and principles for evaluation
Guidance document 8064/VI/97	1997	Details concerning Directive 96/46/EC
Directive 97/57/EC	1997	Substitutes Directive 94/43/EC
Recommendation 1999/333/EC (Annex II)	1999	Quality control measures for monitoring laboratories
Guidance document SANCO/825/00	2000	Substitutes 8064/VI/97 (LC/MS, LC/MS/MS possible)
Guidance document SANCO/3029/99	2000	Details concerning data generation methods

In addition to data requirements and assessment criteria in the context of Annex I listing and the authorization of plant protection products, there are legislative demands for analytical methods addressed to food control and monitoring laboratories. Council Directive 89/397/EEC lays down general principles to be followed by the official food control. Additional measures are stipulated by Council Directive 93/99/EEC. Criteria which should be tested, as far as possible, are described in Annex I to Council Directive 85/591/EEC of 20 December 1985 concerning the introduction of Community methods and analysis for the monitoring of foodstuffs intended for human consumption.[16] Quality control measures are highlighted in guideline 7826/VI/97, which is published as Annex II to the Commission Recommendation 1999/333/EC.[17]

Relevant legal provisions for residue analysis are summarized in Table 2.

3 Evaluation of the submitted methods

3.1 Institutional background

The evaluation of a.i. including the evaluation of the analytical methods is jointly carried out by competent authorities of the Member States and the European Commission. For each a.i., a designated Rapporteur Member State performs the evaluation of the dossier, which is submitted by the applicant and in which all requirements of Annexes II and III to Directive 91/414/EEC must be addressed. The Rapporteur evaluates the data and prepares a draft assessment report (monograph) including a proposal for inclusion or noninclusion in Annex I. The monograph is distributed by the European Commission. Any comments from the Member States and the applicant as well as details of the monograph are discussed in peer review meetings. Issues relating to analytical methods are discussed together with physico-chemical properties in an expert group consisting of about 5–7 alternating scientists named by the Commission as private experts. Their task is to identify problems and to confirm open data requirements. Specific scientific issues may be transferred to the Scientific Committee on Plants. The conclusions of the evaluation of an a.i. are laid down in a Review Report, prepared by the Commission. After consideration by the Standing Committee on Plant Health (since January 2002, the Standing Committee on the Food Chain and Animal Health), a final decision on Annex I inclusion is taken by the European Commission and a Directive is adopted. A detailed description of the whole procedure is given by Wirsing *et al.*[2]

Inclusion in Annex I is the prerequisite for the mutual recognition of authorizations between Member States. At the time Directive 91/414/EEC was adopted in 1991, there were over 800 a.i. authorized for use in the Member States. The goal was to evaluate these at Community level within 12 years. However, the resources necessary to carry out this exercise were not fully recognized when the legislation was adopted. Moreover, time-consuming decision procedures delay the review process. Up to February 2002, 15 existing a.i. and 13 new a.i. were listed in Annex I, whereas 19 a.i. were rejected (see also Table 1). There is clearly a lack of mutual recognition between Member States.

In addition to the evaluation at Community level, Member States have to evaluate the data submitted by applicants, because the authorization of plant protection products

is the responsibility of the individual Member State. It is not possible to apply for authorization at Community level. Therefore, every Member State has established a Competent Authority which may grant authorization (Table 3). For this reason, there are various procedures of data evaluation at Member State level under national legislation and with different institutional backgrounds. Details of the 15 different procedures applied in the Member States cannot be discussed in this article.

3.2 Validation parameters

Validation may mean different things to different people, depending on the context and the application of analytical science. For food control and monitoring purposes, it is generally expected that validation includes the establishment of performance characteristics and evidence that the method fits the respective purpose.[18]

Analytical methods submitted by applicants are evaluated using harmonized criteria (see Section 2.5). The following presentation provides a brief overview of the validation parameters used in the registration of plant protection products and their a.i. These parameters are as follows:

- *Trueness*
  There are various approaches to determine the trueness of methods.[19] The most common is the performance of recovery experiments. According to the guidance document SANCO/825/00,[13] the mean recovery should be in the range of 70–110%. In justified cases, recoveries outside this range can be acceptable.
- *Repeatability*
  Repeatability is defined as precision under conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time. The replicate analytical portion for testing can be prepared from a common field sample containing incurred residues. This approach is used extremely rarely. Normally, repeatability is estimated by the relative standard deviation of recoveries, which should be lower than 20% per commodity and fortification levels according to SANCO/825/00. In justified cases, higher variability can be accepted.
- *Reproducibility*
  Reproducibility in the context of Directive 96/46/EC is defined as a validation of the repeatability of recovery, from representative matrices at representative levels, by at least one laboratory, which is independent of the laboratory which initially validated the study. This independent laboratory may be within the same company, but may not be involved in the development of the method. This concept of independent laboratory validation (ILV) substitutes the conduct of interlaboratory trials (e.g., according to ISO 5725) because the resources are not available taking into consideration the high number of a.i., matrix types and concentration levels which must be validated in the registration procedure.
- *Specificity*
  Specificity is defined in Directive 96/46/EC as the ability of a method to distinguish between the analyte being measured and other substances. According to SANCO/825/00, blank values must be reported using representative matrices. They

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Table 3 Competent authorities for the authorization of plant protection products (status: August 2001)

Authority	Address
Bundesamt und Forschungszentrum für Landwirtschaft Institut für Pflanzenschutzmittelprüfung	Spargelfeldstraße 191, 1226 Vienna, Austria
Ministère des Classes Moyennes et de l'Agriculture Inspection Générale des Matières Premières et Produits Transformés	WTC 3, 8e étage, Boulevard Simon Bolivar 30, 1000 Brussels, Belgium
Biologische Bundesanstalt für Land- und Forstwirtschaft Abteilung für Pflanzenschutzmittel und Anwendungstechnik (BBA)	Messeweg 11/12, 38104 Braunschweig, Germany
Miljoestyrelsen	Strandgade 29, 1401 Copenhagen, Denmark
Ministerio de Agricultura Pesca y Alimentación Subdirección General de Medios de Producción Agricola	Velazuez 147, 28002 Madrid, Spain
Ministère de l'Agriculture Protection des Végétaux	251 rue de Vaugirard, 75732 Paris Cedex 15, France
Plant Production Inspection Centre Pesticide Division	Vilhonvuorenkatu 11 C, V Floor, 00500 Helsinki, Finland
Ministry of Agriculture Directorate of Plant Produce Protection Department of Pesticides	Hippokratus Str. 3–5, 10164 Athens, Greece
Ministero della Sanità Dipartimento per l'Igiene degli Alimenti e della Sanità Pubblica Veterinaria	Piazza Marconi 25, 00144 Rome, Italy
Pesticide Control Service Abbotstown Laboratory Complex	Abbotstown, Castleknock, Dublin 15, Ireland
Administration des Services Techniques de l'Agriculture	16 route d'Esch, BP 1904, 1019 Luxembourg, Luxembourg
College voor de Toelating van de Bestrijdingsmiddelen	Stadsbrink 5, 6700 AA Wageningen, The Netherlands
Centro Nacional de Proteccao da Producao Agricola	Quinta do Marques, 2780 Oeiras, Portugal
Kemikalie Inspektionen	PO Box 13 84, 17127 Solna, Sweden
Pesticides Safety Directorate Mallard House, King's Pool	3 Peasholme Green, York Y01 7PX, UK

should not be higher than 30% of the limit of determination. Moreover, confirmation techniques must be presented in order to avoid false positive results.

- *Limits of determination*
  The limit of determination [or limit of quantitation (LOQ)] is defined in Directive 96/46/EC as the lowest concentration tested at which an acceptable mean recovery (normally 70–110%) and acceptable relative standard deviation (normally <20%) are obtained. The specific requirements for LOQ in crops, food, feed, soil, drinking and surface water, air, body fluids, and tissues are described in Section 4. Because the abbreviation LOD usually means limit of detection rather than limit of determination, the authors prefer not to use this abbreviation here in order to avoid confusion, and LOQ is used throughout. According to Directive 96/46/EC no data with regard to the limit of detection must be given.
- *Applicability*
  As far as is practicable, the methods proposed must employ the simplest approach and commonly available equipment. If possible, standard multi-residue methods should be used. Descriptions of methods must be provided, including all necessary details.

Analytical methods that are submitted by applicants and are assessed at the Community and/or national level are intended to support laboratories involved in post-registration control and the monitoring of food, feed, drinking water, and the environment. Because of the importance of enforcing MRLs, food control laboratories are obliged to conduct quality measures and to employ analytical methods that are validated according to Council Directives 93/99/EEC and 85/591/EEC. These Directives provide only the basic validation parameters and partly the definitions, but contain no further details. Comparing the validation requirements in the context of authorization and those addressed to food laboratories, the definition for reproducibility and the lack of the parameter 'sensitivity' in Directive 96/46/EC proved to be the main differences. Moreover, in the framework of authorization, detailed recommendations were developed. Considerations regarding the connection authorization/food control in the field of residue methods can be found in Lutz Alder's article in this Handbook and in Section 7 of this article.

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4 Requirements for post-registration and monitoring (enforcement) methods

In this section, the general requirements laid down in Directive 96/46/EC[12] and in the guidance document SANCO/825/00[13] are discussed. Furthermore, specific requirements for the different matrices (food of plant and animal origin, soil, water, air, and body fluids and tissues) will be illustrated.

4.1 General requirements

According to Directive 96/46/EC, methods must be capable of determining the a.i. and/or relevant metabolites. For each method and for each relevant representative matrix, the specificity, precision, recovery, and LOQ must be experimentally determined

and reported. These methods must also use the simplest approach, involve the minimum cost, and apply commonly available equipment as much as practicable. The requirement for an analytical method being as uncomplicated and inexpensive as possible cannot be judged in a simple way since it will always be necessary to balance it against the experimental needs given by the purpose. For example, it will probably be impossible to develop a 'simple, low-cost' method if the residue definition contains the parent compound and several metabolites of different polarity. On the other hand, it is not acceptable to develop an enforcement method using sophisticated methodology such as accelerated solvent extraction and quantitation by liquid chromatography/tandem mass spectrometry (LC/MS/MS) if the analyte can be extracted by shaking with an organic solvent and determined by gas chromatography/mass spectrometry (GC/MS) (even if the GC/MS methodology can be regarded as a common technique in general, there is some special instrumentation such as the time-of-flight detector which is not common).

The submitted enforcement method must be applicable in routine monitoring programs. Therefore, it is stated in Directive 96/46/EC that, in principle, residue methods proposed should be multi-residue methods; a standard multi-residue method must be assessed and reported as to its suitability for residue determination. In SANCO/825/00, a scheme of standard multi-methods for different matrices is given. The basis of the multi-methods for food of plant origin involves organic solvent extraction with ethyl acetate[19,20] or acetone (S19 method).[22,23] For soil, water, and air it is also based on the standard multi-methods (see Figure 1). The multi-method scheme is not regarded as a final catalog and may be amended if necessary.

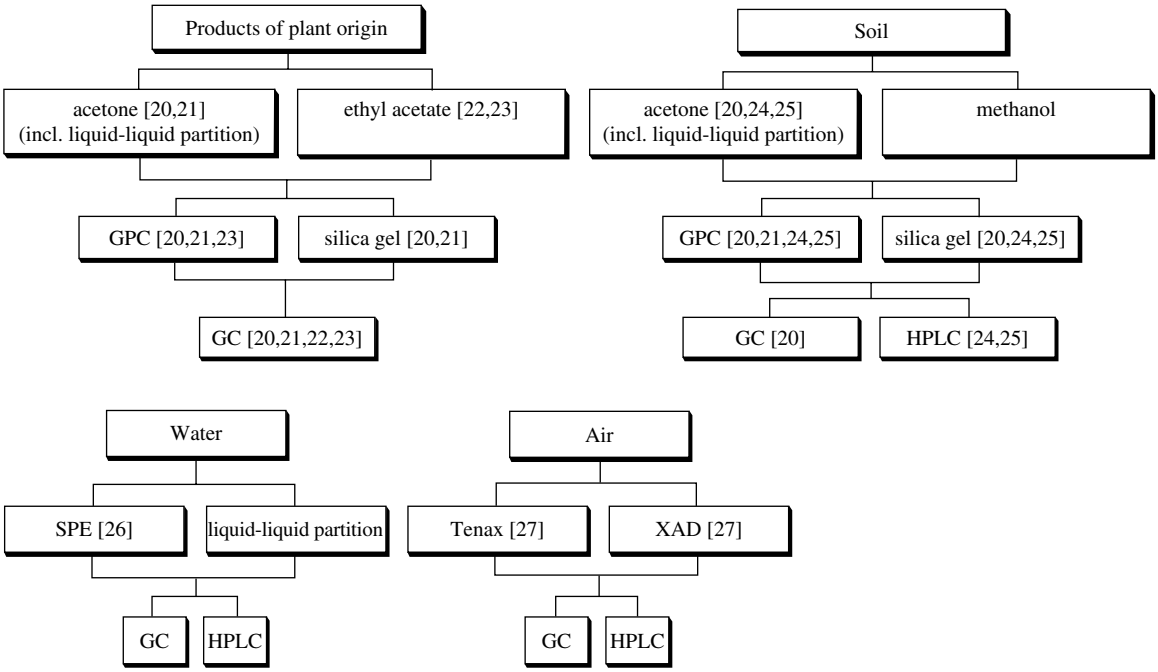


Figure 1 Development/validation approach for multi-residue methods (literature references in brackets)

Owing to the complexity of multi-residue methods for products of animal origin, it is not possible to outline a simple scheme; however, readers should refer to methods described in two references for detailed guidance (Analytical Methods for Pesticides in Foodstuffs, Dutch method collection[23] and European Norm EN 1528.[28]) There is no multi-method specifically designed for body fluids and tissues. The latter matrix can be partly covered by methods for products of animal origin. However, an approach published by Frenzel *et al.*[29] may be helpful (method principle: whole blood is hemolyzed and then deproteinized. After extraction of the supernatant, the a.i. is determined by GC/MS. The LOQ is in the range 30–200 $\mu g\,L^{-1}$, depending on the a.i.).

According to SANCO/825/00, a fully validated method consisting of some or all of the components mentioned above must be reported. Provided that sufficient validation data are published by official manuals, further recovery experiments are not necessary.

If the relevant residue cannot be properly determined using a routine multi-method, an alternative method must be proposed. In the case of residues consisting of a variety of structurally related compounds, a common moiety method may be acceptable in order to avoid the use of an excessive number of methods for individual substances. For example, the relevant residue of isoproturon in plant material is defined as the sum of isoproturon and all metabolites containing the 4-isopropylaniline group. Therefore, residues are determined following hydrolysis as 4-isopropylaniline and are expressed as 4-isopropylaniline equivalents. It is not necessary to validate the method individually for all possible metabolites which are covered by the residue definition (e.g., all metabolites which contain the 4-isopropylaniline group), provided that it is demonstrated that in the first step, the conversion to the common moiety is complete. However, 'common moiety methods' often lack sufficient specificity, and should therefore be avoided if possible. If need be, their use must be justified.

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To avoid different interpretations, a list of analytical techniques, regarded as 'commonly available,' is given in the guidance document SANCO/825/00. Other techniques may also be powerful tools in residue analysis: the acceptance of these additional techniques as part of enforcement methods will be discussed at appropriate intervals by governmental experts. Therefore, whilst not wishing to prevent

Table 4 Validation parameters and criteria applied for the assessment of enforcement analytical methods

Specificity	Blank values must be reported: they should not be higher than 30% of the LOQ. Confirmatory method/technique must be described if appropriate
Recovery	The percentage of the analyte originally added to a sample of the matrix which contains no detectable level of the analyte (the normally accepted range of the mean recovery is 70–110%)
Precision:	
Repeatability	Relative standard deviation of recoveries lower than 20% per representative matrix and fortification level
Reproducibility	Confirmation of the results by at least one independent laboratory
Limit of quantitation (LOQ)	Lowest concentration at which an acceptable mean recovery is obtained with a relative standard deviation $\leq$20%

development, the list will be discussed and if necessary updated. The current list is valid until 31 December 2003 and contains at present the following techniques:

- GC: nitrogen–phosphorus detector (NPD), flame photometric detector (FPD), electron capture detector (ECD), flame ionization detector (FID), mass-spectrometric detector (MS)
- high-performance liquid chromatography (HPLC): ultraviolet (UV), diode-array detection (DAD), tandem mass spectrometry (MS/MS), fluorescence detector, electrochemical detector
  (column switching)
- atomic absorption spectrometry (AAS)
- immunoassay methodology.

Because the validation of the last technique requires a different approach to chromatographic and spectrometric methods, several important points are described in SANCO/825/00 which should be taken into account when such methods are used. The authors do not wish to go into detail on this subject, since on the one hand very few methods have been submitted up to the present, and on the other hand it would go beyond the scope of this article.

The submitted enforcement method must be described in detail along with specifying equipment, materials and conditions. The following points must be addressed:

- introduction, including definition of the analyte(s) and scope of the method
- outline/summary of method, including validated matrices, LOQ and range of recoveries and fortifications
- apparatus
- reagents (including manufacturer and purity as well as full details of standard compound purity and associated method of determination or clear reference of origin, if commercially available)
- sample preparation
- procedure (extraction, cleanup, derivatization, determination)
- calculation (including typical calibration curves, linearity, correlation coefficient r)
- evaluation (specificity, recoveries, LOQ, repeatability)
- important points and special remarks in analysis (e.g., matrix-dependent deviation, reagent stability)
- clearly labeled representative chromatograms of matrix blank and standard as well as fortified samples (at the LOQ) and/or spectra; where possible, representative chromatograms and/or spectra of incurred samples should be submitted, but it is not necessary to submit the complete set of raw data; labeling should include sample description, chromatographic scale, and identification of all relevant components in the chromatogram
- hazards or precautions required
- references.

As mentioned above, the specificity, precision, recovery, and LOQ must be experimentally determined and reported for each method and for each relevant representative matrix. In Table 4 brief explanations are given to describe the validation parameters in

the context of 96/46/EC and the practical approach in SANCO/825/00 (for a definition of these terms, see also Section 3.2).

The general sample set for method validation parameters is the same for all matrices under consideration (except body fluids and tissues, see Section 4.2.5):

- LOQ 5 samples
- 10 times LOQ or relevant limit (set or proposed) when the limit is higher than 10 times LOQ 5 samples
- control 2 samples

Confirmatory techniques must be submitted if the analytical method is not highly specific. A confirmatory method will not be required if the original method uses GC/MS, provided that at least three fragment ions with an m/z ratio of >100 are used for identification/quantitation. The rationale for the selection of the ions monitored should also be provided. When a confirmatory method/technique is required to demonstrate specificity, the properties of the analyte should be considered when deciding on an appropriate method/technique. In SANCO/825/00 acceptable confirmatory techniques are specified as follows:

- HPLC/DAD, if the UV spectrum is characteristic; in this case a UV spectrum obtained under the conditions of determination must be submitted
- alternative chromatographic principle (e.g., substitution of HPLC by GC) from the original method
- alternative detection method
- derivatization, if it was not the first-choice method
- different stationary and/or mobile phase of different selectivities.

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In addition, variation of partitioning and/or cleanup steps can be useful for confirmation in special cases.

The extent of validation of confirmatory techniques is currently under consideration. One approach is that the extent of validation may be smaller than for the enforcement method. In principle, validation in triplicate at the relevant concentration level (LOQ or MRL) is sufficient. In the case where an MRL is set for multiple crops, a single validation in all representative crop groups is sufficient. A confirmatory method for residues in air is not required if a corresponding method was submitted for the other sample matrices. This approach is realized in Germany.[30]

4.2 Specific requirements

4.2.1 Food of plant and animal origin

The enforcement method must be suitable for the determination of all compounds included in the residue definition in order to enable Member States to determine compliance with MRLs. It is not feasible to validate a method for all commodities if a wide range of MRLs are set. Therefore, a concept of crop groups was developed in SANCO/825/00. The following crop groups with representative crops are presented:

- cereals and other dry crops (e.g., barley, wheat, rye)
- commodities with high water content (e.g., lettuce, cucumber)

- commodities with high fat content (e.g., rape seed, linseed, olives)
- fruits with high acid content (e.g., lemons, grapefruits).

For each group, one representative sample matrix has to be used for method validation. If the intended use is restricted to one of the crop groups, the method must be validated only for this group. On the other hand, the method has to be validated for all groups if the use is intended for a variety of crops that belong to two or more different groups. In addition, specific crops which are difficult to analyze due to matrix interference require individual method validation (e.g., hops, brassica varieties, bulb vegetables, herbs, tea).

There is some discussion within the Member States aimed at method validation for all crop groups in every case in order to support the enforcement of MRLs established for other crops. Additionally, detailed lists of the crop groups are under development. For example, it seems to be that almost all fruits can be classified as 'fruits with high acid content' (exception: e.g., bananas and certain varieties of apples). Depending on the variation of the analytical method necessary to obtain acceptable results, it may be possible to cover more than one group by validation using one crop. For example, if the validation is performed with lemons and the pH value has no influence on the recovery of the a.i., it may be acceptable to waive the validation using a representative commodity with a higher water content.

Validation of the analytical methods for food of animal origin has to be performed with milk, egg, meat, and fat. The latter is required only if log $P_{O/W}$ is >3 and metabolism studies indicate significant residues in fat, because in this case it is likely that an MRL will be set. Other tissues such as kidney or liver must be validated only if an MRL is set or proposed for these tissues. The issue of the general necessity of analytical methods for food of animal origin is not addressed in Directive 96/46/EC or SANCO/825/00. At this moment, the Working Group 'Pesticide Residues' proposes an MRL on a case-by-case basis. However, a pragmatic approach is presented in SANCO/825/00.

According to Directive 96/68/EC,[31] an analytical method for the determination of residues in food of animal origin is not required when metabolism study in animals is not required. On the other hand, according to Point 6.4 of the Directive, where a feeding study is required, an analytical method for the determination of residues in products of animal origin must be submitted. In other cases, the requirement for an analytical method depends on the establishment of an MRL for food commodities of animal origin.

Two additional requirements are specific to the analysis of residues in food. The first requirement depends on the LOQ to be achieved (see Table 5).

Table 5 Relation between the maximum residue limit (MRL) and the limit of quantitation (LOQ)

MRL ($mg\,kg^{-1}$)	LOQ ($mg\,kg^{-1}$)
>0.1	≤0.1
0.1	≤0.05
0.05	≤ 0.02
<0.05	≤MRL × 0.5
Set at LOQ	LOQ

The second requirement is that enforcement methods for food must be validated by an independent laboratory [independent laboratory validation (ILV)]. The sample set is identical with the general sample set (see Section 4.1). If the method is identical for all four crop groups (mentioned at the beginning of the section), it may be sufficient to perform the ILV for plant materials with a minimum of two matrices, one of them with a high water content. In the case of food of animal origin, the ILV should be performed with at least two of the matrices: milk, egg, meat, and, if appropriate, fat.

The prerequisite that the laboratory chosen to conduct the ILV trials must not be involved in the method development and/or in its subsequent use is not applicable for multi-methods. If the applicability of a multi-method is published in an official manual,[20,23,32] an ILV is not obligatory for this particular a.i. ILV is always required for single methods. Communications between the chosen laboratory and the method developers must be reported, provided that these communications were required to carry out the analysis successfully. Also, any subsequent amendments or modifications to the original method must be reported. Furthermore, the ILV report must contain a statement as to the applicability of the method. In contrast, it is not necessary to confirm the results of the enforcement methods for soil, water, body fluids, tissues, and air by an independent laboratory validation.

4.2.2 Soil

The proposed LOQ for the analysis of residues in soil is related to the impact on nontarget organisms and to phytotoxic effects. Generally, the proposed limit of determination should not exceed 0.05 mg kg^{-1}. For certain a.i., however, the required sensitivity may not be technically achievable. For example, the LOQ for some sulfonylurea herbicides must be below 0.05 μg kg^{-1} because of the extremely low effect concentrations of this class of a.i. However, at present a reliable chromatographic/spectrometric analysis of these a.i. below 0.05 μg kg^{-1} is not available. Bioassays used as screening tests may be useful to exclude the occurrence of residues from phytotoxic compounds. Unfortunately, these methods are incapable of giving accurate measurements of the level of the active substance present or necessarily identifying which a.i. is present, but can give a rough guide as to whether biologically active levels of pesticides are present.

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At present no a.i. is known to have an unacceptable impact on nontarget organisms assessed in the authorization procedure in the concentration range below 0.05 mg kg^{-1}.

For certain naturally occurring nontoxic a.i., an enforcement is not sensible (e.g., lecithin, rape seed oil). Analytical methods for residues in soil are not necessary if the DT_{90} values of the a.i. and relevant metabolites are less than 3 days (e.g., fosetyl), because in general, the results from residue analyses are not meaningful if the a.i. is rapidly degraded.

4.2.3 Water (including drinking water, groundwater, and surface water)

From the analytical point of view there is no essential difference between drinking water and groundwater. Therefore, it is sufficient if the enforcement method is validated only for either drinking water or groundwater. The LOQ for drinking water/groundwater must be $\leq$0.1 μg L^{-1} (EU drinking water limit).[10]

Table 6 Limits for different species

Organism	Acute test	Long-term test
Fish[a]	LC_{50}	NOEC[b]
Daphnia[a]	EC_{50}	NOEC
Algae	EC_{50}	–
Higher aquatic plants	EC_{50}	–

[a] Guidance on whether the values from the acute or the long-term test should be used is given in the EU-Guideline 8075/VI/97.[33] Normally, the values of the long-term test are relevant for residue analytical purposes.
[b] NOEC, no observable effect concentration.

In the case of surface water, the LOQ must not exceed a concentration which has an impact on nontarget organisms deemed to be unacceptable according to the requirements of Annex VI.[11] At present, no harmonized limits for surface water exist. Therefore, provisions in Annex VI of Directive 91/414/EEC will be used to calculate guidance limits for analytical methods for surface water. In SANCO/825/00 the limits given in Table 6 are established [the relevant concentrations (the lowest will always be taken into consideration) depend on the species as indicated and can be taken from toxicity tests].

For certain naturally occurring nontoxic a.i. an enforcement is not sensible (e.g., lecithin, rape seed oil). Analytical methods for residues in water are not required if the DT_{90} values of the a.i. and relevant metabolites are less than 3 days (e.g., fosetyl) because, in general, the results from residue analyses are not meaningful if the a.i. is rapidly degraded.

4.2.4 Air

Methods to determine the a.i., and/or relevant metabolites in air during or shortly after the application must be submitted unless it can be justified that exposure of operators, workers, or bystanders does not occur. In SANCO/825/00 it is stated that spray drift and particle-associated as well as gaseous substances have to be taken into consideration because both can cause relevant exposure of operators, workers, or bystanders. Therefore, an analytical method must also be submitted for relevant substances with a low vapor pressure ($<10^{-5}$ Pa).

The LOQ must take into account relevant health based limit values or relevant exposure levels. In SANCO/825/00 a method to calculate a relevant health based limit is given. The limit of quantitation must be equal to or lower than the concentration C, which is defined by equation (1).

$$C = \frac{\mathrm{AOEL_{inhalative}} \times 0.1 \times 60}{20}\ [\mathrm{mg\,m^{-3}}] \tag{1}$$

where

0.1 = safety factor
60 = body weight in kg
20 = air intake [volume per day in m^3].

$AOEL_{inhalative}$ can be substituted by the $AOEL_{systemic}$. In the case that neither acceptable operator exposure level (AOEL) values are available, the proposed or established acceptable daily intake (ADI) value can be considered.

The methods must be suitable for identifying both particle-associated and gaseous residues. It is sufficient to quote literature proving that the sorbents used also adsorb particle-associated residues. The sorbent material retention capacity must be determined. This should be carried out by determining the recovery rates of the a.i. and/or metabolite fortified on the sorbent at a defined air temperature and relative humidity, after the passage of a defined air volume for at least 6 h. The breakthrough volume or the maximum tested capacity (micrograms of substance per adsorption tube) without breakthrough must be reported.

4.2.5 Body fluids and tissues

Analytical methods for the determination of residues in body fluids and tissues must be submitted only if the a.i. is classified as toxic or highly toxic. The method has to be validated only at the LOQ: in general blood 0.05 mg L^{-1} and tissues 0.1 mg kg^{-1} (meat or liver, if not investigated under food of animal origin, see Section 4.2.1).

It is indispensable to consider the metabolism pathway of an a.i. for the development of an analytical method.

5 Requirements for data generation methods

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Reliable residue data are generated during the development of an a.i. to support the assessment of the consumer risk (residue data and toxicological data) and the impact on the environment (fate and behavior, efficacy and ecotoxicological data). It is critical that these analytical methods are reliably validated. In the guidance document SANCO/3029/99 rev. 4 (11/07/00),[14] harmonized requirements for the residue analytical method are described. Validated analytical methods are required for the following studies:

Residue studies

- supervised trials and animal feeding studies for consumer risk assessment, setting of MRLs
- processing studies
- stability of residues during storage

Environmental fate and behavior

- field dissipation, accumulation, laboratory degradation or sorption studies (non-radiolabeled) for parent and major/significant environmental metabolites (usual matrices of interest are soil, water and sediment)

Efficacy

- for soil: carry over of phytotoxic levels of the a.i. and/or biologically active metabolites
- for water: assessment of effectiveness of procedures for cleaning spray equipment

Ecotoxicology

- verification of the actual exposure levels to a.i. and major/significant metabolites in ecotoxicity tests (usual matrices of interest are soil, water, sediment, and feedstuff)

Toxicology

- dietary and gavage nonradiolabeled studies and air-inhalation studies

Operator or worker exposure

- dosimetry, inhalation, and biological samples.

In the following section the general requirements specified in SANCO/3029/99 are described and discussed. Following this, specific requirements for the different matrices such as food of plant and animal origin, soil, water, air, and body fluids and tissues are illustrated.

5.1 General requirements

The majority of validation data required for analytical methods supporting authorization purposes are common to those described for enforcement methods (see Section 4). However, some of the requirements such as 'minimum cost' and 'commonly available' equipment do not apply to methods supporting pre-registration studies (e.g., the use of GC/MS/MS technology).

Full descriptions of validated methods must be provided, including details of equipment, materials, and conditions used as described in Section 4.1. In addition, the following items must be addressed/apply:

- sample storage, where validation samples have been stored prior to analysis (conditions of storage, e.g., temperature and storage interval)
- general sample preparation techniques (including sample sizes and numbers of samples)
- interpretation of chromatograms (where appropriate)
- determination of extraction efficiency.

In contrast to the requirements for enforcement methods, validation of a previously collaboratively tested method, which is used to generate data, should be validated for new laboratory conditions. Also, where published methods are submitted, validation is required, when applied to the relevant sample matrix and laboratory conditions.

Analytical methods must be capable of determining the a.i. and/or relevant metabolites in the presence of the sample matrix. Where the sample contains more than one isomer, analog, etc., of an a.i. or relevant metabolite, the method should distinguish between individual isomers/analogues where this is necessary for carrying out risk assessment.

The sample set must include two fortification levels appropriate to the proposed LOQ and likely residue levels or 10 times the LOQ, except for body fluids and tissues (considered in Section 5.2.3) where validation data at the LOQ are sufficient. Five determinations should be made at each fortification level. In general, mean

recoveries for each level should be in the range 70–110% and the relative standard deviation (RSD) should be ≤20% per level. In certain justified cases, higher level variability may be accepted. Lower recoveries may be acceptable for matrices which are difficult to analyze and for difficult analytes, provided that precision data are acceptable.

Contrary to the enforcement methods, additional confirmatory analysis is not necessary where it is demonstrated that the primary residue method is specific to the analyte(s) and the source of the analyte(s) is known.

The use of common moiety methods acceptable in exceptional circumstances where there is no other practical means of determining the target analyte, and in these cases, full justification is required. This should include an explanation of why the compound cannot be determined by a specific analytical technique. For existing a.i., common moiety methods are also acceptable, in cases where the residue definition includes a common moiety. Moreover, validation data must be presented separately for all relevant components.

The use of immunoassay methodology for residue trial analysis is in principle just as acceptable as for enforcement methods, provided that the method has been adequately validated. Because the validation of such methods requires a different approach, as opposed to chromatographic and spectrometric methods, some important points to be aware of in the use are explained in SANCO/3029/99. The authors do not go into detail on this subject here, since on the one hand very few methods have been submitted up to the present, and on the other it would go beyond the scope of this article.

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5.2 Specific requirements

5.2.1 Plants, plant products, foodstuffs (of plant and animal origin), and feedingstuffs

In contrast to the requirements for enforcement methods and to ensure sufficient quality of the generated data, validation data should be submitted for all types of crop samples to be analyzed. However, matrix comparability and a reduced validation data set may be considered where two or more very similar matrices are to be analyzed (e.g., cereal grain). A reduced sample set may also be acceptable (two levels, at least three determinations and an assessment of matrix interference) provided that the investigated samples belong to the same crop group as described in SANCO/825/00 (see also Section 4.2.1).

In the case of products of animal origin, validation should be performed, where appropriate, with milk, liver, kidney, muscle, fat, and egg.

Validation should be carried out for each component of the residue definition in each sample matrix used for risk assessment purposes.

In general, a nonspecific method is not acceptable because it is possible for the identity of the source of the analyte to be called into question. However, in cases where derivatization from a common species is the only method available (e.g., dithiocarbamate compounds), the use of a nonspecific common moiety method may be acceptable.

5.2.2 Soil, water, sediment, and air samples

The method must be capable of determining all components (a.i. and relevant/major metabolites) that are included in the residue definitions used in the assessment of risk to nontarget organisms. For ground (drinking) water and air, the risk to consumers/operators or bystanders must also be considered.

In the case of soil and sediment, the proposed LOQ should not exceed 0.05 mg kg^{-1}. If the phytotoxic concentration in soil for sensitive crops or the toxic concentration for nontarget organisms is lower than 0.05 mg kg^{-1}, the LOQ has to be lower than these values. For water, the proposed LOQ should not exceed 0.1 μg L^{-1} for ground (drinking) water and should take into account for surface water the lowest end point from aquatic toxicity studies or, where relevant, the lowest phytotoxic level. The LOQ for surface water must be less than the lowest chronic NOEC for either fish or *Daphnia* or the EC_{50} for algae. If no chronic data must be generated, the LOQ must be less than the lowest acute EC_{50}/LC_{50} for fish or Daphnia.

The conditions for validation of an analytical method for the determination of residues in air are the same as the requirements given in Section 4.2.5.

5.2.3 Body fluids and tissues

The matrices to be validated depend on the target/purpose of the study, e.g., blood, urine, muscle, or liver. The latter two may be covered by methods developed for food of animal origin. The method must take into account all relevant compounds used in the assessment of risk to consumers/operators or bystanders. The required LOQ depends on the toxicological end point of interest.

6 Availability of analytical methods

Pursuant to Council Directive 91/414/EEC, a plant protection product shall not be authorized by Member States unless its residues can be determined by appropriate methods. In order to ensure residue control both by governmental and private institutions, analytical methods must be available for all enforcement laboratories. Therefore, the confidentiality which is generally granted for information submitted by industry does not apply to analytical methods for post-registration control and monitoring purposes (Article 14). Nevertheless, the provision concerning data protection has to be followed by the Member States. In granting authorizations, they may not make use of analytical methods put at the enforcement laboratories' disposal for the benefit of other applicants, unless an agreement was made by the first applicant in this regard, or unless the data protection periods have expired (Article 13).

In principle, the laboratories concerned may ask the competent authorities in their countries (Table 3) for analytical methods, but national legislation and national practice should be taken into consideration.

As a special service, the German authority has published reviews on residue analysis concerning new a.i. contained in plant protection since 1996, including selected physical-chemical data. Recoveries obtained in fortification experiments and LOQs for analytical methods for determination in crops, food of plant and animal origin,

soil, water, and air are presented. Furthermore, relative retention times and mass spectrometric data are reported.

The BBA publishes reviews of analytical methods for existing a.i.[34] References and a table of a.i. which can be determined using the standard multi-method S19 or its new modular version[32] are presented on the World Wide Web.[35]

Methods submitted by industry are partly used for implementation in national collections of analytical methods (e.g., in the German Method Collection of §35 LMBG). This activity often involves a modification of the analytical procedure and extended validation. Some examples for this approach are discussed by Lutz Alder in this Handbook.

7 Perspectives

The analytical methods for post-registration control and monitoring purposes submitted by industry to the authorization bodies help the enforcement laboratories. Data requirements from the authorization procedures are constantly compared with the needs of enforcement laboratories, in order to supply them with relevant data, and to avoid the generation of superfluous information.

In this context, the list of commonly available techniques and the list of obsolete dangerous reagents must be revised regularly. Furthermore, questions which are asked frequently by applicants should be responded to, e.g., lists of commodities for the four crop groups and extent of data for confirmation techniques.

Moreover, new technologies such as LC/MS/MS should be considered and their potential should be recognized in the future. Currently food control laboratories monitor only a part of the pesticides used in their routine work. They prefer active ingredients that can be analyzed by multi-methods or some group-specific methods, because resources to check all relevant pesticides are normally not available. Therefore, many a.i. are monitored only on a case-by-case basis or not at all. An LC/MS multi-residue method, which may be developed in the future, could cover this gap to a large extent.

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The activities of enforcement laboratories should not be focused on irrelevant problems. Therefore, a clear definition of the relevant residue is needed. In the crops and food sector, procedures are well established to derive the two residue definitions, one for risk assessment and one for monitoring, from metabolism studies. As far as environmental samples are concerned, there is much potential for improvement. There are no clear criteria as to which metabolites should be included in monitoring and control programs. Additionally, the development of criteria for nonpriority pesticides, e.g., naturally occurring compounds or low-risk products, which can be excluded from monitoring exercises would be helpful for laboratories and evaluators.

In the future, the enforcement of feedingstuffs will be more important because the MRLs established for food become partly obligatory for feed also. Validation concepts for this matrix must be developed in collaboration with laboratories obliged to control feedingstuffs, considering the approach of four matrix types for food crops mentioned in Section 4.

A project for the future could be the comparison of the data sets required by authorities of countries such as the USA or Japan. Moreover, discussions can be

expected on whether or not components of the Food and Agriculture Organization (FAO)/International Atomic Energy Agency (IAEA) report on method validation practices[36] should be integrated into authorization requirements, regarding the existing legal framework.

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References

1. European Commission, Directive 91/414/EEC, *Off. J. Eur. Commun.*, No. L 230, 19.08.1991, 1. (1991). This Directive and also the following ones are also available on the World Wide Web: **http://europa.eu.int/eur-lex/en/index.htm**.
2. B. Wirsing, J.M. von Kietzell, H. Kula, C. Landsmann, D.F. Flynn, and J.-R. Lundehn, *Nachrichtenbl. Dtsch. Pflanzenschd.* **52**, 164 (2000).
3. European Commission, Directive 76/895/EEC, *Off. J. Eur. Commun.*, No. L 340, 09.12.1976, 26 (1976).
4. European Commission, Directive 86/362/EEC, *Off. J. Eur. Commun.*, No. L 221, 07.08.1986, 37 (1986).
5. European Commission, Directive 86/363/EEC, *Off. J. Eur. Commun.*, No. L 221, 07.08.1986, 43 (1986).
6. European Commission, Directive 90/642/EEC, *Off. J. Eur. Commun.*, No. L 350, 14.12.1990, 71 (1990).
7. European Commission, Directive 89/397/EEC, *Off. J. Eur. Commun.*, No. L 186, 30.06.1989, 23 (1989).
8. European Commission, Directive 93/99/EEC, *Off. J. Eur. Commun.*, No. L 290, 24.11.1993, 14 (1993).
9. **http://europa.eu.int/comm/food/fs/pp/pp_index_en.html.**
10. European Commission, Directive 98/83/EC, *Off. J. Eur. Commun.*, No. L 330, 05.12.1998, 32 (1998).
11. European Commission, Directive 97/57/EC, *Off. J. Eur. Commun.*, No. L 265, 27.09.1997, 87 (1997).
12. European Commission, Directive 96/46/EC, *Off. J. Eur. Commun.*, No. L 214, 23.08.1996, 18 (1996).
13. European Commission, Guidance Document on Residue Analytical Methods, SANCO/825/00/rev. 6, 20.06.00. European Commission, Brussels (2000). This guidance document and also the following ones are available on the World Wide Web: **http://europa.eu.int/comm/food/fs/ph_ps/pro/wrkdoc/index_en.htm**.
14. European Commission, Residues: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (Part A, Section 4) and Annex III (Part A, Section 5) of Directive 91/414, SANCO/3029/99 rev. 4, 11.07.00. European Commission, Brussels (2000).
15. European Commission, Guideline Developed with the Standing Committee on Plant Health with Regard to the Applicability of Good Laboratory Practice to Data Requirements According to Annexes II, Part A and III, Part A of Council Directive 91/414/EEC, 7109/VI/94 Rev. 6, 14.07.1995. European Commission, Brussels (1995).
16. European Commission, Directive 85/591/EEC, *Off. J. Eur. Commun.*, No. L 372, 31.12.1985, 50 (1985).

17. European Commission, Recommendation 1999/333/EC, *Off. J. Eur. Commun.*, No. L 128, 21.05.1999, 25 (1999).
18. J.D. MacNeil, J. Patterson, and V. Martz, 'Validation of analytical methods – proving your method is "fit for purpose," in "Principles and Practices of Method Validation," ed. A. Flajgelj and A. Ambrus, MPG Books, Bodmin, pp. 100–107 (2000).
19. R. Hoggerbugge and P. van Zoonen, 'Validation of analytical data in a research and development environment,' in "Principles and Practices of Method Validation," ed. A. Flajgelj and A. Ambrus, MPG Books, Bodmin, pp. 19–28 (2000).
20. 'Manual of Pesticide Residue Analysis,' ed. H.-P. Thier and H. Zeumer, DFG Pesticides Commission, VCH, Weinheim, Vols. I and II (1987).
21. W. Specht, S. Pelz, and W. Gilsbach, *Fresenius' J. Anal. Chem.*, **353**, 183 (1995).
22. A. Anderson and H. Pålsheden, *Fresenius' J. Anal. Chem.*, **399**, 365 (1991).
23. 'Analytical Methods for Pesticide Residues in Foodstuffs,' Ministry of Public Health, Welfare and Sport, Amsterdam, 6th edn. (1996).
24. *Agribiol. Res.*, **46**, 155 (1993).
25. G. Offenbächer, 'Bestimmung von Herbiziden in Böden mittels HPLC mit UV-Detektion,' in "Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik," ed. R. Bassler, VDLUFA-Verlag, Darmstadt, Vol. VII, pp. 1–25 (1996).
26. 'Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlamm-Untersuchung, physikalische, chemische, biologische und bakteriologische Verfahren,' Fachgruppe Wasserchemie i.d. GDCh mit dem Normenausschuß Wasserwesen (NAW) im Deutschen Institut für Normung eV (DIN), VCH, Weinheim and Beuth Verlag, Berlin, Vol. V, Gruppe F: Gemeinsam erfaßbare Stoffgruppen (1996).
27. M. Blacha-Puller, J. Goedicke, K.-H. Leist, H.-G. Nolting, J. Pauluhn, D. Pick, R. Pfeil, K. Riegner, and J. Siebers, *Nachrichtenbl. Dtsch. Pflanzenschutzd.*, **46**, 60 (1994).
28. European Norm (EN) 1528, Fatty Food – Determination of Pesticides and Polychlorinated Biphenyls (PCBs). European Committee for Standardization Beuth Verlag, Berlin (1997).
29. T. Frenzel, H. Sochor, K. Speer, and M. Uihlein, *J. Anal. Toxicol.*, **24**, 365 (2000).
30. H. Kohsiek, *Bundesanzeiger*, Nr 232, 09.12.2002, 23090 (2002).
31. European Commission, Directive 96/86/EC, *Off. J. Eur. Commun.*, No. L 277, 30.10.1996, 25 (1996).
32. 'Amtliche Sammlung von Untersuchungsverfahren nach §35 LMBG,' Untersuchung von Lebensmitteln, Modulare Multimethode zur Bestimmung von Pflanzenschtuzmittelrückständen in Lebensmitteln, Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV), Beuth-Verlag, Berlin (1999).
33. European Commission, Guidance Document on Aquatic Ecotoxicology, 8075/VI/97 rev. 7, 08.07.2000 (Draft), European Commission, Brussels (2000).
34. R. Hänel, R. Fischer, and J. Siebers, *Nachrichtenbl. Dtsch. Pflanzenschd.*, **53**, 1 (2001).
35. **http://www.bba.de/analytik/analytik.htm**.
36. 'Guideline for Single-laboratory Validation of Analytical Methods for Trace-level Concentrations of Organic Chemicals,' in "Principles and Practices of Method Validation," ed. A. Flajgelj and A. Ambrus, MPG Books, Bodmin, pp. 179–252 (2000).

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Regulatory considerations for residue analysis and methods on crops and food: the approach of Japan

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1 Background

In 1947, the Japanese government promulgated the Pesticide Registration Law (PRL) administered by the Japanese Ministry of Agriculture and Forestry [now Ministry of Agriculture, Forestry and Fisheries (MAFF)]. At that time, the purpose of this law was to control the quality of end-use pesticide products since there were many poor-quality and fake products in the market in the 1940s.

In 1972, the government amended the PRL and required that applicants obtain a toxicological set of data for registration of pesticides, since the environmental and toxicological concerns regarding pesticide residues in agricultural products and other toxicological issues regarding residual chemicals had become apparent since 1947. In 1984, MAFF introduced requirements of Good Laboratory Practice (GLP) guidelines on toxicological studies. Since then, MAFF has extended the data requirements of the GLP guidelines to metabolism studies and effects on aquatic animals, etc., to protect both humans and the environment. When applicants intend to register a pesticide for use on edible crops or paddy fields, the authority establishes the registration withholding limit (RWL) of the product on crops and in water. After registration, if the authority finds residues in crops or water over the RWL, it will request the registrant to revise their Good Agricultural Practice (GAP) procedures or revoke the registration of the pesticide.

The Food Sanitary Law in Japan, which is regulated by the Ministry of Health, Labor and Welfare (MHLW), has established maximum residue limits (MRLs) and monitors the residue levels in food commodities in the market, including both domestic and

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imported products. Local governments also have surveillance schemes for crops in the domestic market.

When MRLs are set under Japanese law, the Japanese authorities will consider the Codex MRLs for international harmonization. However, Japan has set original food consumption amounts and GAPs. In this respect, the Japanese regulatory agency retains the rights to establish original values for the MRLs for each commodity based on toxicological exposure assessment.

To commercialize agricultural end-use products in Japan, applicants are required to submit appropriate data to MAFF. The data required for registration are summarized in this section.

MAFF requires the following data sets. Except for field studies for biological evaluation and residue sample preparation, studies including toxicological and physicochemical studies must be conducted according to GLP guidelines under Japanese regulatory law. The Japanese authorities accept toxicological data that are generated by applicants in foreign countries that are conducted under the GLP system. However, some data, such as crop residue trial data, bioefficacy, results of soil degradation studies, etc., on the compounds, should be generated under Japanese regulatory systems for registration in Japan.

The data for the registration of active ingredients required by MAFF are summarized as follows:

Toxicity studies:

- acute toxicity study (oral, dermal, and inhalation)
- irritation study (skin, eyes)
- skin sensitization
- acute neurotoxicity study
- 90-day oral toxicity study
- teratogenicity study
- reproduction study
- chronic toxicity study [dog (1 year), rat (2 years)]
- carcinogenicity study
- mutagenicity study.

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Biological studies:

- efficacy and phytotoxicity studies.

Metabolism studies:

- plant
- soil
- animal.

Residue studies:

- crop
- soil
- water.

Physico-chemical properties
Effects on aquatic animals and plants

Table 1 Classification of crops

Plant group	Principal crop
Rice	Rice
Grain and sugarcane	Wheat, barley, rye, corn, buckwheat, sugarcane
Fruits (except citrus and melons)	Peach, loquat, kiwi, apple, pear, persimmon, nectarine, apricot, cherry, UME, strawberry, grape, ginkgo nut, chestnut, walnuts
Citrus fruits	Unshu MIKAN, large-size citrus fruits, small-size citrus fruits
Vegetables (include melon)	Bell pepper, okra, shishitou, pumpkin, cucumber, tomato, eggplant, watermelon, melon
Leaves and flower plants	Cabbage, Chinese cabbage, Japanese radish (leaves), broccoli, komatuna, soybeans (immature), field pea, kidney beans (immature), onion, garlic, scallion, hops
Root or stem plants	Japanese radish (root), carrot, ginger, potato, sweet potato, taro, sugar beet
Beans, oil plants	Soybean (dry), azuki bean (dry), pea (dry), broad bean (dry), rapeseed, sesame seed, safflower
Mushrooms	Shiitake mushrooms, enokidake mushrooms
Tea	Tea

2 Plant metabolism studies

Plant metabolism studies will provide information on the absorption, translocation, dissipation and degradation of the agrochemical. This information defines the residual analytes of regulatory concern that could include either the parent compound or metabolites in the field crops. Plant metabolism studies should be conducted with at least three crop representatives of three different crop groups listed in Table 1. One of the major objectives is to determine the comparative metabolism of the agrochemicals between animals and plants among different plant species. MAFF approves metabolism studies that are conducted in foreign countries, which should be operated under the certified GLP system.

The test substance is a pure chemical compound, which may or may not be labeled with isotopes such as ^{14}C, ^{13}C, ^{3}H, ^{2}H or ^{35}S.

Test plants are grown under controlled conditions similar to actual cultivation conditions.

The formulated product of the test substance should be prepared as a representative formulation for registration (or a formulation of similar composition), and applied to the test plants according to the use pattern indicated in the documents for registration. If several different use patterns are indicated in the documents for registration, studies should be conducted on each.

Samples are collected at the usual harvest time for each crop. However, when it is very difficult to identify the metabolic pathway on the sample owing to the long preharvest intervals, samples should be collected several times between first application and the usual harvest time.

The collected plant samples are analyzed as soon as possible after harvest. When samples or their extracts need to be stored, appropriate storage conditions are imperative. The stability of metabolites should be monitored during the storage period.

Metabolites, including their conjugates [not less than 10% of the total recovery ratio (TRR)], should be identified or characterized chemically along with their conjugates.

3 Residue studies on crops

In preparation for a registration submission, applicants should conduct a residue study on each edible crop through supervised field trials. Residue data should be prepared for each use pattern and formulation type to be labeled.

3.1 Residue analytical method

The applicants must provide the analytical methods used for the determination of the residues in the supervised field trial(s). The following features of the analytical methods are required by MAFF:

- should have the ability to determine the parent and all defined residues of regulatory concern;
- has to be specific for separating interfering substances from the target analytes;
- should provide standard deviation within 10% at nominal residue levels and 20% at the limit of quantitation (LOQ) level;
- should provide a fortified recovery ratio between 70 and 120% at two different concentrations, such as at the LOQ level and above the maximum residue level of the field trial;
- should provide a limit of analytical quantitation about one-tenth of the MRLs or of assumed residue level;
- should provide an analytical level of 0.01 mg kg^{-1} or below the LOQ when residues are not detectable;
- normally, analytical methods are required to provide an LOQ level in the range 0.01–0.05 mg kg^{-1}.

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3.2 Preferred methodology for conducting supervised field trials

3.2.1 Planning phase

(1) Crop and crop grouping. Residue studies have to be conducted with each target crop proposed for registration; one variety of the target crop is acceptable.

The citrus and cereal crop groups can be represented by any crop within that group (below), with the exception of Unshu orange for citrus, and rice, corn and buckwheat for cereals. Data from Chinese orange can cover grapefruit, navel orange, etc., and wheat and barley can cover oat and rye.

(2) Site/location selection. Residue studies are conducted at two or more sites for each target crop. Trial sites should be selected from typical growing areas for the target crop and should include different environmental conditions that might affect the levels of pesticide residues.

(3) Test materials. Residue studies are conducted with each formulation for which it is intended to seek registration; some formulation types are acceptable for extrapolation. Classification of pesticide formulations in Japan is as follows: dusts, granules, wettable powders, water-soluble powders, water-dispersible granules, dust–granule mixtures, tablets, emulsion concentrates, oil solutions, liquid formulations, suspension concentrates, microcapsules, emulsions, suspo-emulsions, micro-emulsions, smoking agents, fumigants and aerosols.

(4) Use pattern. Residue studies are conducted according to the use pattern of the recommendation on a label or a proposed recommendation on the label of each respective pesticide application method for which a registration will be required: foliar application, seed treatment, seedling treatment, seed tuber treatment, seed spray application, seed dressing, smoking, fumigation, broadcast treatment (soil incorporation), row treatment (soil incorporation), furrow treatment (soil incorporation), pricking-in hole treatment (soil incorporation), plant foot treatment (soil incorporation), drench and injection.

(5) Raw agriculture commodity requirements. Residues on all parts of crops that can be consumed are analyzed without preparation (e.g., washing and peeling, etc.) in the residue studies. There are no requirements on the residue data on processing foods.

(6) Residue decline study requirements. All residue studies should provide a decline study that shows the dissipation pattern of the residues for at least three different sampling times and shows the MRL. Typical sampling times are suggested to be a pre-harvest interval (PHI) of 1, 3, 7, 14, 21, 28 (30), 45, 60 days, etc. Especially for daily harvested crops such as tomato, cucumber, eggplant and bell pepper, a PHI of 1 day should be selected.

(7) Sample preparation by contract research organization. In Japan, GLP for field residue study work has not yet been established and sample preparation for residue studies by private companies is not authorized. Contract research organizations are limited to prefectural research institutes and MAFF-recognized local research institutes, mainly neutral organizations, such as the Japan Plant Protection Association (JPPA).

The JPPA has its own research institute in Ibaraki prefecture and two experimental stations in Kochi and Miyazaki for conducting many types of research services to evaluate the performance of agrochemicals. The research institute and experimental stations have test fields, greenhouses and research laboratories with the capability for chemical analyses. The research institute also provides several services such as identification of virus diseases or other crop pests on plants.

3.2.2 Site preparation

The following considerations are critical in the selection and preparation of residue study test sites:

- Trial sites should be selected in the growing areas of the target crop, from at least two different areas. Test sites must be far enough apart to avoid cross-contamination.

- Test sites are selected from areas with different environmental and cultural conditions that might affect the levels of pesticide residues (e.g., temperature, soil characteristics, planting patterns).
- Some crops are grown in limited production areas, such as hops and sugar beets in Hokkaido. Trials on these crops can be conducted at two sites in the same area.
- Trial sites should not be selected at locations where soil contamination with test substances could be anticipated or where similar types of agrochemicals have been used within 1 year.
- Trial sites must be selected on the basis of uniformity (soil characteristics, areas of sunshine, etc.), and areas that are susceptible to erosion or areas where drift of chemicals from neighboring fields might occur should be avoided. Irrigation sources are supplied, preferably nearby.
- Control plots should be placed upslope and upwind of the treated plots.
- Buffer zones should be set up between plots to avoid contamination.
- Plot size should be large enough to apply the test material, to obtain more than twice the required samples (most crops require a total of 12-kg sample weight) and to be able to use appropriate harvesting equipment.
- Two separate greenhouses should be used for smoking agents or fumigant studies in order to avoid contamination.
- The number of fruit trees required for a field residue trial may be as small as a single tree, but it is often more than one tree per plot. Two treatments may not be applied to the same fruit tree. If crops are grown both in greenhouses and in an open field, residue studies must also be conducted in a greenhouse.
- The crop variety will be selected from among the most commonly cultivated varieties at the trial site and production area.
- Cultivation methods must be the same as in normal local commercial practice.
- Limited pesticide must be applied to protect the crops from disease and insect pests in order to cultivate healthy crops, according to recommendations for pest control. However, crop protection agents that could interfere with the residue analyses must be used.
- Nets or fences should be set up around the plots to protect them from bird and animal damage, if necessary.
- Field maintenance must be done first in the control and next in the treated plots. All documents must be entered in the field notebook.

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Field notebooks that scientists are using at JPPA contain the following items:

- name of the trial agrochemical and lot numbers;
- test site information (soil characters, style or materials of greenhouse, etc.);
- summary of cultivation methods;
- list of records of cultivated crops and maintenance agrochemical used in that plot within 1 year;
- list of records of maintenance agrochemical used in the trial period.

Record of application of agrochemicals:

- plot size, date of application, type of sprayer, number of nozzles, concentration, spray volume, calculation method, adjuvant used, application method, mixing time, spraying time, weather conditions, stage of crop, etc.;

- record of sampling: sample number, date of sampling, weight of sample, sampling time, weather, equipment used for sampling, method of sampling, date of shipping, method of shipping, address of analytical laboratory;
- map of the trial field.

3.2.3 Test materials

- End-use formulation products are used as test materials in the field residue study.
- Test materials are supplied by the sponsor company.
- As the test materials are received, the following items should be recorded: Date of reception, name of the agrochemical sponsor company, name of the test material, condition of the test material, lot number, amount, etc.
- Test materials are stored under the conditions indicated on the label. Temperature and humidity for storage of test materials should be monitored during the course of the trials.
- Test materials are returned to the sponsor company after the trial is finished.

3.2.4 Application phase

Spray solution is prepared as follows:

For solid test materials:

- The names of test materials and plot numbers of the test sites are entered on the plastic bags that will contain the test materials.
- Amounts of test materials are weighed in the plastic bag with an electric balance that must be certified annually.
- The amount of water to dilute the test materials is measured into a plastic bucket.
- Aliquots of water used for dilution are taken from plastic bucket and divided into other plastic buckets. A portion of the water is poured into the plastic bags containing the test materials. After thorough agitation, the concentrated test substance solution is poured into the plastic bucket and the plastic bag is rinsed twice with the rest of the water. After thorough agitation, the diluted test solution in the bucket is poured into the application equipment.

For liquid test materials:

- The amount of water with which to dilute the test materials is measured into a plastic bucket. Amounts of test materials are measured with a pipette, syringe or graduated cylinder and poured into the plastic bucket. After thorough agitation, the diluted test solution in the bucket is poured into the application equipment.
- Application equipment must be of a standard (commercial) type.
- The types of sprayer and nozzle and number of nozzles are selected depending on the required spray volume and the shape of the crop canopy. Application method, maximum spray volume, application frequency and interval must be as stated in the protocol.
- Spray volume should be based on the stage of growth of the plant. Spray volume is calculated based on plot size shape and stage of growth of the crop.

- Values of the spray volume and amount of test substance are rounded off to one decimal place.
- Spray volume is reported as liters per 10 acre (10 a).
- Pesticide must be sprayed uniformly on foliage and fruit.
- Pesticide should not be sprayed during strong winds, and especially when wind is blowing towards the control plot from the treated plot.
- In the case of rainfall during, soon after or before spraying or before the spray solution is dried on treated crops, the field investigator must contact the sponsor company and follow instructions.
- After the application, the application equipment should be washed twice with running water.
- All documents must be entered into the field notebook.

3.2.5 Sampling phase

- Standard (commercial) sampling equipment should be used to simulate commercial harvesting of the crop. Sampling should be carried out according to the harvest/sampling methods that are common to local commercial practice. The crop fraction is the same as the commercial type, e.g., apple fruit with stem, onion bulb without foliage, and leafy vegetables without root are recommended for sampling.
- The collected sample weight must be more than 4 kg in most crops at each sampling time. For light crops such as strawberry, 2 kg or more should be collected. The number of individual units sampled is more than 10 for crops such as watermelon, melon, cabbage, Chinese cabbage, radish, and pumpkin.
- To avoid contamination of the samples, the sampling equipment should be free from contaminants and a sampling bag must be used to protect the samples from contamination.
- Plot numbers and sampling dates are recorded on the sampling bag.
- Samples must be collected representing the entire plot by avoiding sampling along the border areas and following zigzag, X or S patterns.
- In the case of grain, samples are harvested from the entire plot, and care must be taken to avoid contamination of surrounding plots.
- Samples must be collected first from the control and then from the treated plots starting with the lowest concentration and finally from those applied at the highest concentration.
- Control samples are collected once on the day of the first sampling of the treated plots in most studies to avoid contamination.
- Control and treated samples must be put in separate bags.
- Abnormally grown or damaged crops should not be collected (e.g., infected by insects or diseases, unripe, too big or too small, touching the ground).
- Surface residues must not be removed during sampling.
- Soil adhering to root and tuber crop samples must be removed with cold running water but samples should not be scrubbed with a brush. The samples should be dried in a clean room to avoid contamination.
- Cereals are dried in a greenhouse after sampling and shipped within 4 weeks of harvest.

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- Sampling date, PHI, weight of sample and part of the crop sampled must be recorded as stated in the protocol.
- All documentation for sampling must be entered in the field notebook.

3.2.6 Storage and shipping

- The collected samples are divided equally into two portions in a clean room that is free from contamination for shipping to an official analytical laboratory and a private analytical laboratory.
- Uncontaminated cardboard boxes and (news)papers must be used for packaging.
- Plastic bags cannot be used to protect from rot owing to high temperatures and humidity during shipping.
- Newspapers are suitable as wrapping material to protect the samples from decomposition during shipping.
- Each plot sample must be placed in a separate cardboard box.
- Samples must be packed/unpacked starting with control samples to the samples applied with the test compounds in order from the lowest to the highest concentration.
- Surface residues must not be removed during packing.
- Trial site, name of crop, name of agrochemical tested, and sample number are recorded on the outside of the cardboard boxes.
- A shipping card is placed in each cardboard box with the samples, and the original copy of the shipping card is kept at the trial site.
- An original copy of the shipping card is attached to the final report to the JPPA. The shipping card contains the following items: name of trial site, name(s) of field investigator(s), name of crop and variety, method of cultivation (open field or greenhouse), name of agrochemical (formulation type), application method (concentration and spray volume), application frequency, application date, sampling date, sample number, remarks, and date of reception at the analysis laboratory.
- Samples must be shipped to two analytical laboratories immediately after packing. Most samples are shipped by door-to-door delivery service at a temperature of 5 °C. All documents must be included in the field notebook.

3.3 Field data (field report) presentation

Copies of the final sampling reports must be submitted to the headquarters of JPPA and two analytical laboratories, immediately after the trial is finished. The original of the final sampling report should be archived at the trial site.

The applicants will submit the reports of the final sampling and the analytical result to MAFF. The final sampling report must contain the following items:

- name of the agrochemical;
- experimental code number, type of formulation, name of formulation product, and concentration of the active ingredient, lot number, etc.;
- name of target crop and variety;
- name of trial site and address;

- name of field investigator;
- type of soil texture (e.g., light clay, sandy loam, sandy clay loam, clay loam) and water loss in depth (cm day^{-1});
- list of crops grown and agrochemical used within 1 year in the test field;
- summary of cultivation: grown in the greenhouse or in an open field, date of seeding, date of planting, date of transplanting, crop spacing, plant density, planting pattern, date of fertilizer application, kind and quantity (per 10 a) of fertilizer, time and date of harvest;
- crop growing stage at treatment;
- list of agrochemicals used for pest control during trial period;
- name, concentration, spray volume (liters per 10 a) and date of application of agrochemicals;
- trial plot; plot size and number of crop plants (even for rice), size of greenhouse (height, area and capacity), map of the trial field;
- method of application: date of application, spray solution concentration, spray volume (liters per 10 a), method of application, stage of crops, type of sprayer, start time of application, weather situation during application, adjuvants, remarks;
- sampling: date of sampling, time of sampling and weather conditions, order of sampling, shipping weight of sample, date of shipping, method of sampling, method of packing, addresses of analytical laboratories, method of shipping, remarks;
- weather report;
- location of recording and sampling data and documentation;
- temperature (average temperature recorded at 1-h intervals);
- precipitation (duration and amount during a day, from 0:00 to 24:00).

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3.4 Extrapolation among the formulation types

Basically, MAFF requests that the official field residue trials test combinations of each use pattern and formulation type intended for commercialization. However, MAFF may approve/accept data generated by extrapolating the data from one formulation type to certain other types, as shown in Table 2. Some formulation types will be able to extrapolate to the other formulation types. Table 2 shows that the data obtained using the formulation types in the left column can be extrapolated to those of formulation types in the right column.

3.5 Residue definition

To select and define the target analytes for the residue analysis of crops in a field trial, applicants should consider metabolites/degradation products of the test materials by conducting plant and animal metabolism studies and by assessing toxicity of the metabolites/degradation products.

MAFF proposes that 10 crop groups be considered for the plant metabolism studies: (1) rice, cereals and sugarcane, (2) fruit (except citrus), (3) citrus, (4) fruiting vegetables, (5) leaf and flower vegetables, (6) root and tuber vegetables, (7) beans, (8) oilseed, (9) mushrooms, and (10) tea. MAFF requests three plant metabolism studies of three different crop groups among the 10 crop groups noted above.

Table 2 Extrapolation of formulations

Formulation types tested	Formulation type for extrapolation
Emulsion concentrate (EC)	EC
Wettable powder (WP)	EC, SC, WP
Suspension concentrates (SC)	SC
Dust (D)	EC, SC, WP, D
Granules (G)	G
Liquid (L)	L, WS, WSG
Flow dust (FD)	FD, D, WP, WDG, SC, EC, EW, CE
Aerosol	Aerosol, L, WS, EC, EW, CE
Smoke	Smoke
Paste	Paste
Water-soluble (WS)	WS, L, WSG
Water-soluble granules (WSG)	WSG, WS, L
Emulsion in water (EW)	EW, CE, EC
Concentrated emulsion (CE)	CE, EW, EC
Micro-emulsion (ME)	ME, L, WS
Micro-capsules (MC)	MC

Almost 10% of the TRR level of the metabolites/degradation products will be selected and defined as the analytes for residue analysis.

The toxic effects of selected plant analytes will be assessed by comparison with the toxicities of similar metabolites found in animal metabolism studies. The amount of the analytes reported in the plant metabolism study is one of the important factors used to establish the residue definition.

4 Market basket survey in Japan

Under the Food Sanitary Law, MRLs are established on each crop. The Law requires that the residual levels of agrochemical in the crops in the market must be under the MRLs. There are 217 active ingredient MRLs established as of September 2001.

The MHLW recognizes the official analytical methods for inspection and survey of MRLs for crops in the market; 112 residue analytical methods have already been established and authorized. MHLW also approves the use of the original analytical methods for the official surveillance by which methods local governments analyze residues as they inspect local commercial farm commodities. In such a case, the reliability of the original analytical methods should be guaranteed as being equivalent to that of the official analytical methods.

The official methods were validated for conducting residue analysis in government laboratories. Some methods are applicable to multi-residue analyses for organophosphorus compounds, organochlorine compounds, *N*-methyl phenylcarbamates, pyrethroids and related nitrogen compounds. Single analysis methods are also established that, in many cases, use high-performance liquid chromatography, and metabolites are often included as part of the residue definition. Normally, the history of agrochemical usage in the crops is unknown. Multi-residue analytical methods are

useful and efficient for inspection under the Food Sanitary Law, if such methods have sufficient accuracy and precision.

5 Conclusion

It is critical for applicants to obtain registration with the Japanese MAFF before the end-use products of agricultural chemicals start to be sold in Japan. The applicants should submit appropriate data sets from toxicological studies, residue trial studies on the crops, bioefficacy data, etc., to the MAFF under the PRL. During the registration evaluation process, registration-withholding limits will be established by the PRL. During field trials, applicants should conduct the trial under the official or semi-official field conditions, and follow the guidance that is requested by authorities. The Food Sanitary Law establishes MRLs for each crop. The MHLW and local governments will survey the raw commodities in the domestic market and imported products.

Further reading

Notifications by Director General No. 12 – Seisan 8147, Ministry of Agriculture, Forestry and Fisheries, Tokyo (2000).
Notifications by Director General No 13 – Seisan 1739, Ministry of Agriculture, Forestry and Fisheries, Tokyo (2001).
Notifications by Director General No. 13 – Seisan 3986, Ministry of Agriculture, Forestry and Fisheries, Tokyo (2001).
Notifications by Director General No. 13 – Seisan 3987, Ministry of Agriculture, Forestry and Fisheries, Tokyo (2001).

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General approaches for residue analytical method development and validation

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1 Introduction

Analytical chemistry is an important field in the life sciences whether the main focus is health (pharmaceutical chemistry), nutrition (food chemistry), food supply (pesticide chemistry), environment (water chemistry, waste minimization, disposal or treatment) or lifestyle (textiles, mobility, cosmetics). Thus chemists (and other scientists) working analytically, whether they are trained originally as analytical chemists or whether they come from a different field and use analytical chemistry as support for their research area, play an important role in supporting the progress in the life sciences.

Each chemist working analytically uses (sometimes without any awareness) the analytical process, a scheme (see Figure 1) by which most analytical problems are assessed. The analytical process is a multi-step approach to solving questions by analytical chemistry and includes the following steps:

- Define the problem and the question(s) to be answered. These may originate in any field of the life sciences, or in any technical or scientific area, or even in politics or society.
- Define the analytical approach, such as the material and the analytes to be looked for so as to (possibly) answer the questions asked and to solve the problems.
- Select an appropriate analytical method, with definition of its purpose and utility. If none of the available methods fits the analytical purpose, try to deduce method approach(es) from existing methods for structurally related compounds or materials by introducing carefully selected modifications and adaptations.
- Other considerations could include availability of reagent(s) or equipment, method for routine analyses vs limited samples, and confirmatory method vs multi-residues.
- Plan for method validation and/or analytical quality control.
- Define the specimen(s) and the sampling procedure(s) to obtain a representative sub-sample of the materials to be examined.

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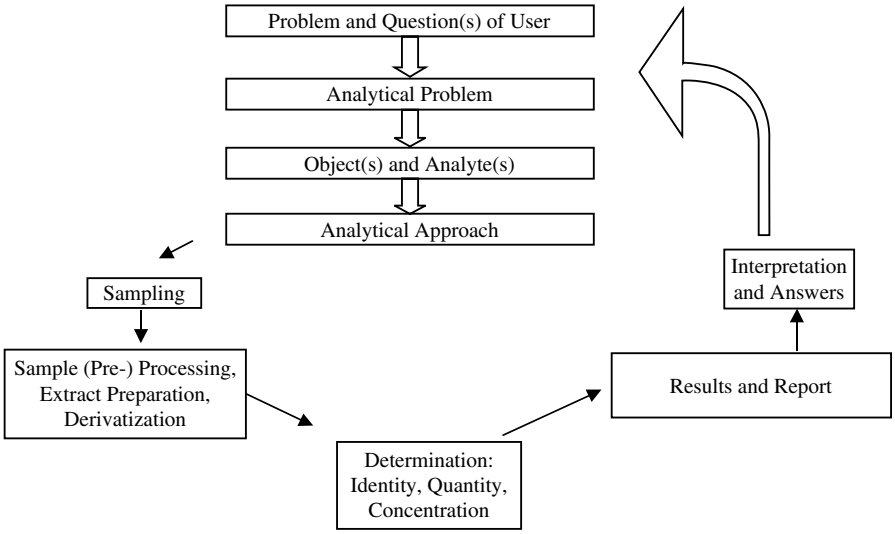


Figure 1 The analytical process

- After sampling, homogenization, extraction, cleanup, concentration and possible derivatization, use a suitable determination method which provides sufficient selectivity or specificity and sufficient sensitivity.
- Ensure that the analytical methodology gives reliable results in terms of identity (absence of false-positive findings) and of absence (no false-negative findings) of the analyte(s). This requires processing of concurrent analytical quality control samples.
- Reliable identification may require confirmation by a method with different selectivity or employing a different analytical principle.
- Ensure that quantitation yields accurate and precise results by monitoring the background, recoveries and standard deviations.
- Report the results in a comprehensive manner to allow interpretation of the findings and the drawing of conclusions.
- Answer the questions and solve the problem posed at the beginning of the analytical process.

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The analytical chemist is not involved in the entire analytical process in all cases. It is always preferable, however, not only to focus on the analytical method, but also to consider the background of the analytical task and the consequences of the analytical results.

2 Approaches to analytical method development

2.1 Properties of the analyte(s)

The structure of the compounds to be analyzed is usually known, but there are cases where unknown metabolites, degradation products, conjugates or species (e.g.

metal complexes, adducts) need to be searched for. This is the case, for example, in metabolism or degradation studies, or when (unwanted or positive) effects are presumably caused by compounds of unknown identity.

The following physico-chemical properties of the analyte(s) are important in method development considerations: vapor pressure, ultraviolet (UV) absorption spectrum, solubility in water and in solvents, dissociation constant(s), n-octanol/water partition coefficient, stability vs hydrolysis and possible thermal, photo- or chemical degradation. These valuable data enable the analytical chemist to develop the most promising analytical approach, drawing from the literature and from his or her experience with related analytical problems, as exemplified below. Gas chromatography (GC) methods, for example, require a measurable vapor pressure and a certain thermal stability as the analytes move as vaporized molecules within the mobile phase. On the other hand, compounds that have a high vapor pressure will require careful extract concentration by evaporation of volatile solvents.

A UV spectrum with a pronounced absorption above 210 nm allows UV detection after liquid chromatography (LC), but an absorption maximum in the range of visible light may also decompose during cleanup procedures and require the elimination of light when handling extracts.

Water solubility, dissociation constant(s) and n-octanol/water partition coefficients allow one to predict how an analyte may behave on normal-phase (NP), reversed-phase (RP), or ion-exchange solid-phase extraction (SPE) for sample enrichment and cleanup.

2.2 Functional groups of the analyte(s)

The presence of heteroatoms usually provides a convenient feature for improving selectivity by employing selective detection mechanisms. GC may then use: flame photometric detection (FPD) for S and P atoms and to a certain extent for N, Se, Si etc.; thermoselective detection (TSD) and nitrogen–phosphorus detection (NPD) for N and P atoms; electron capture detection (ECD) for halogen atoms (F, Cl, Br, and I) and for systems with conjugated double bonds and electron-drawing groups; or atomic emission detection (AED) for many heteroatoms.

The isotopic patterns of $^{35/37}Cl_n$ or $^{79/81}Br_m$ atoms present in the analyte molecule are very helpful when mass spectrometry (MS) is used for specific detection and identification after GC or LC. The presence of several halogens in a molecule, however, may decrease the sensitivity in normal resolution (quadrupole or ion trap) MS and tandem mass spectrometry (MS/MS) due to the presence of an isotopic ion pattern, but when high-resolution mass spectrometry (HRMS) (magnetic field HRMS) detection is employed (using the sum of the mass defects of the halogen atoms present in a fragment ion), the selectivity and consequently sensitivity are improved tremendously. Multiple fragmentation ions are formed in the electron ionization (EI) source when labile bonds break, whereas chemical ionization (CI) or electrospray ionization (ESI) sources result in softer ionization and thus less bond breakage and a limited number of ions with less fragmentation.

On the other hand, electronegative substituents such as F, Cl and Br atoms, but also NO_2 and COOH moieties, are extremely sensitive when negative ions and

fragments are monitored by gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS).

Functional groups and reactive moieties may cause losses by adsorption on the matrix and surfaces. On the other hand, functional groups may be used to increase the selectivity and sensitivity of the methods by derivatization, forming distinct derivatives with different properties during cleanup and chromatographic separation and detection. Examples are the esterification of carboxylic acid moieties (using, e.g., diazomethane, diazoethane, trimethylsilyldiazomethane, sulfuric acid–methanol or butanol), silylation of carboxylic acid, hydroxyl or amino moieties, benzylation of OH moieties, cyclization of two neighboring moieties (using, e.g., acetylacetone or aceticanhydride), etc. Precolumn derivatization of functional groups is mandatory if GC is employed for polar or thermolabile analytes. Postcolumn derivatization after high-performance liquid chromatography (HPLC) may drastically improve selectivity as UV detection can be substituted by fluorescence detection.

2.3 Properties of the sample material

The composition, properties and size (weight, volume) of the sample material to be analyzed are important aspects for analytical method development and for analyte enrichment vs depletion of sample matrix.

Sampling of air to determine worker exposure or for environmental purposes usually includes the easy task of eliminating first the air (N_2, O_2, trace gases) and then the humidity present in the air (water which may condense and saturate adsorption columns). Particles (e.g. salts, soil, soot) present in the air and trapped during air sampling may also contain active species or adsorptive surfaces and thus cause losses of the analytes. Large volumes (liters to cubic meters, high-volume sampling up to 1000 m^3) of air, however, are usually sampled by eliminating air and water without losing the analytes, thus reducing the sample size by a large factor. These procedures depend on the vapor pressure and the adsorption or absorption mechanisms that retain the analyte.

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Sampling of water for monitoring purposes allows sample sizes of 1–3 L or less. Eliminating water and retaining the analyte are relatively easy for organic molecules with a high n-octanol/water partition coefficient, but become more difficult for analytes with ionic properties and high solubility in water. If the water sample contains a high load of salts, silt or organic matter such as surface (river, pond, sea) water, possible adsorption on filtered matter needs to be considered.

Plant material water contents range from high (>90%, e.g. vegetables) to low (<10%, e.g. straw, herbs, tea, hops, etc.). Thus the ratio between the analytes (residues) and the organic matter potentially interfering with the analysis is very different for, e.g., cucumber and camomile tea. Other ingredients in plant materials such as acids, oil, sugars, starch or substances typically for the taste and effect of plant materials may have properties similar to those of the analytes and thus interfere in or influence the cleanup procedures.

Materials of animal origin such as tissue, fat, milk, egg or blood contains usually relatively large amounts of fat, proteins and carbohydrates that need to be reduced during cleanup to allow enrichment of the analytes to be searched for.

Sample size may influence the analytical approach, e.g. 0.1 L of milk is easily obtained and extracted, but 10 mL of blood should be sufficient for monitoring purposes, and 5 g of fat is already the upper limit for an efficient fat cleanup by partition or gel permeation chromatography (GPC).

2.4 Availability and practicality of analytical instrumentation

Analytical instrumentation ranges from that for generally available techniques such as liquid chromatography/ultraviolet detection (LC/UV) or gas chromatography/flame ionization detection (GC/FID) with their limitations with regard to selectivity and sensitivity on the one hand, to very sophisticated techniques such as GC, LC or capillary electrophoresis (CE) coupled to triple-quadrupole tandem mass spectrometry (MS/MS) in space, ion trap MS^n in time, time-of-flight (TOF), or high resolution (HRMS) mass spectrometers on the other. The availability of the very sophisticated (and expensive) instrumentation (such as GC with HRMS, or LC with triple-quadrupole MS/MS) may lead to the over-use of this instrumentation with very little attention given to cleanup and chemistry, whereas the less sensitive/selective techniques require one to focus more on the laboratory procedures of the method.

2.5 Consideration of time, throughput, ruggedness and quality

In the development of analytical methods one has to consider also cases where a fast response is required, e.g. clinical and forensic chemists or toxicologists need methods which yield results in a few minutes or hours to allow a fast response in cases of poisoning. In this event, accurate quantitative results may be of less importance, but the time from sampling to result may be lifesaving, whereas the throughput (i.e. number of analyses per day) is not so much of concern.

Residue analysts working in enforcement laboratories are required to analyze specimens collected in the market or obtained from import/export facilities in time to exclude any goods with unacceptable residues being sold or imported. Their analytical problem is focused mainly on the presence or absence of regulated residues where they need to avoid any false-positive or false-negative results. Hence for these analysts the analytical method needs to give them reliable results in a day or two or before the foodstuff of plant or animal origin is sold, consumed or spoiled. On the other hand, they are required to collect and analyze large numbers of representative samples as tolerances or maximum residue levels (MRLs) need to be surveyed and enforced.

Throughput may also be an issue when monitoring programs for groundwater or for characteristic consumer/market baskets yield very large numbers of samples for analysis. Such monitoring programs are expected to yield reliable results and therefore require special care in terms of accuracy and precision of the results. This is often ensured by frequent and rigid quality assurance measures such as intra- and inter-laboratory comparison tests and the use of certified reference materials.

An analytical method can be considered rugged when it can be transferred from one laboratory to another with comparable experience without much effort in adaptation to the different technical personnel and to the different equipment and instrumentation.

The above leads to the concept of the quality of an analytical method (not to be confused with the quality of the results). A method should be as simple or as sophisticated as necessary to serve its purpose in yielding reliable results and in answering the questions posed at the beginning of the analytical process.

3 Practical examples

3.1 Extending the scope of the multi-residue method DFG S19

The multi-residue method DFG S19[1–3] was intended to be used in state enforcement laboratories or in private contract or food industry laboratories. It was aimed initially only at plant materials and water and included a relatively large number of pesticides which are amenable to GC.

Its principles include polar extraction with acetone–water (2 : 1, v/v), homogeneous partitioning of the target molecules into an organic solvent, GPC cleanup on Bio-Beads, fractionation by adsorption column chromatography on silica gel (SiO_2) deactivated with 1.5% water and finally GC with various selective detection methods (NPD, ECD, FPD).

Confirmation of suspected residue findings relies on the various chromatographic principles of cleanup and determination (GPC, NP-LC, GC), and is further supported by re-analysis of the final extract(s) on a GC stationary phase of different polarity, providing modified selectivity, or by the use of GC with specific mass spectrometric detection [GC/MS or gas chromatography/tandem mass spectrometry (GC/MS/MS)].

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The practicality of the method was further improved by introducing a one-beaker extraction and partition step, dichloromethane being replaced with ethyl acetate–cyclohexane as the organic phase during homogeneous partitioning. These solvents together with the acetone portion of the extraction form the upper organic phase, whereas the hydrophilic matrix constituents remain in the aqueous phase saturated with sodium chloride.

The method procedures are very time-efficient by always using a well defined portion of the extracts, thus avoiding multiple extractions, time-consuming rinses and overloading of the chromatographic cleanup systems with co-extracted sample matrix.

For oily crops such as nuts and oilseeds, a slightly different extraction procedure with 10% acetone in acetonitrile is used.

The scope of the multi-residue method is extended permanently by testing and then including further active substances that can be determined by GC. Acidic analytes (such as phenoxyacetic acids or RCOOH metabolites) are included into the homogeneous partitioning by acidifying the raw extracts to a pH below the pK_s value of the carboxylic acids. To include these analytes in the GC determination scheme they have to be derivatized with diazomethane, diazoethane, trimethylsilyldiazomethane, acidic esterification or benzylation, or by silanizing the COOH moiety.

Another extension of the DFG S19 method was achieved by applying it successfully to foodstuffs of animal origin such as whole milk and egg, muscle meat, offal, fat and honey. Depending on water and fat content, either water–acetone (e.g. for milk, meat, possibly egg and honey) or acetone–acetonitrile (e.g. for offal, egg, fat) solvent extraction is preferable. When high fat or oil contents in the raw extract are expected,

an additional hexane–acetonitrile partitioning/wash step prior to GPC reduces the load on the size-exclusion GPC column and thus avoids overloading the pores, which can cause a shift of the analyte retention times to earlier elution.

With the use of ion trap GC/MS for determination, the method became more universal when the ion trap mass spectrometer was operated in the full-scan mode, providing for many analytes sufficient sensitivity and a high specificity by providing full mass spectra. With quadrupole GC/MS operated in the selected ion monitoring (SIM) mode, or with ion trap GC/MS operated in the selected ion storage (SIS) mode, the sensitivity of the method was further improved, but the universal multi-residue character of the DFG S19 method, however, was reduced to a target method still very useful for the confirmation of suspect residue findings. A further improvement was achieved by using ion trap or quadrupole GC/MS/MS for determination of target analytes in the final extracts.

As the sensitivity and selectivity of the above GC/MS methods are for many analytes around 1 pg μL^{-1} injected into the GC system, cleanup by SiO_2 fractionation can be omitted when larger sample sizes (25–100 g) are possible. For difficult dry (e.g. hops, pharmaceutical herbs) or oily (e.g. rape seed, fat, liver) materials which start with smaller sample sizes (5–10 g) and tend to overload the chromatographic cleanup systems, however, cleanup is still an important requirement as the GC injection system is vulnerable when the ratio of co-extracted material to analyte is too high.

A further extension of the DFG S19 method was achieved when polar analytes and those unsuitable for GC were determined by LC/MS or more preferably by liquid chromatography/tandem mass spectrometry (LC/MS/MS). Triple-quadrupole MS/MS and ion trap MS^n have become more affordable and acceptable in the recent past. These techniques provide multiple analyte methods by employing modes such as time segments, scan events or multiple injections. By improving the selectivity and sensitivity of detection after HPLC separation, the DFG S19 extraction and cleanup scheme can be applied to polar or high molecular weight analytes, and cleanup steps such as SiO_2 fractionation or even GPC become unnecessary.

What can be achieved by the fully extended DFG S19 approach is impressively demonstrated by the residue analysis of many pesticides during minor crop registration. Considering an analyte such as azoxystrobin, which is tested for application in minor crop cultures or pharmaceutical herbs such as artichokes, peppermint, camomile or St. John's Wort, GC is expected to result in problems of thermal degradation or unacceptable tailing of its large and polar molecule, and LC/MS/MS becomes the method of choice. Further, as pharmaceutical herbs or teas are very dry materials, only a small sample size, e.g. 5–10 g, can be extracted. This small size of the analytical samples is still acceptable and considered sufficiently representative if taken from a larger quantity previously homogenized frozen with dry-ice. To circumvent possible matrix effects (e.g. precipitation) in the final extracts for LC injection, atmospheric pressure chemical ionization (APCI) and ion trap MS^n detection of positive ions, the raw extract obtained by homogeneous partition is fractionated by GPC, which in this case reduces the presence of larger biomolecules. A further improvement in specificity without loss in sensitivity is achieved by employing an ion trap MS^3 method. Once this target method has been established for a few different plant materials, it can be easily extended to related analytes such as pyraclostrobin or most other plant materials.

Another successful adaptation of the fully extended DFG S19 approach is the determination of, e.g., fenpyroximate in all type of berries by LC/MS/MS with APCI monitoring of positive ions directly in the S19 raw extract, and further the determination of trifluralin by LC/MS/MS with APCI monitoring of negative ions after performing a short SPE cleanup on an ion-exchange material. Similar approaches have used GC/MS/MS for, e.g., fenpropimorph and kresoxim methyl in St. John's Wort and peppermint.

Once several target methods employing, e.g., LC/MS/MS techniques have been combined, a multi-residue method will evolve which includes the DFG S19 extraction procedures in combination with the generally applicable GPC cleanup and requires automatic multiple injections to circumvent the limitations of the limited HPLC peak capacity and the target-specific MS/MS methods.

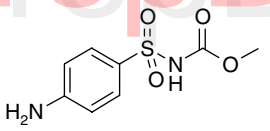
3.2 What can go wrong?

The above examples can be extended to the majority of older and newer active substances described in, e.g., 'The Pesticide Manual' [4] and to numerous relevant metabolites featuring hydroxyl or carboxyl moieties or even for conjugates; however, there remain various active substances and metabolites that still require careful and extensive method development.

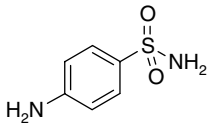
The following are some of the main pitfalls one can expect:

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- Ionic or amphotheric character of the analytes, e.g. asulam and its metabolites acetylasulam, sulfanilamide, acetylsulfanilamide:



Asulam



Sulfanilamide

The carbamate –NH– moiety present in asulam has acidic properties (e.g. the pK_a value for asulam is 4.82). On the other hand, the $-NH_2$ moiety present in sulfanilamide has a slightly alkaline character. Considering these properties, the partition of these analytes into an organic solvent should depend strongly on the pH value in the aqueous phase.

Such analytes require carefully chosen extraction conditions in terms of pH, solvent composition and technique. Also, these analytes tend to become lost by adsorption on (glass) surfaces or undergo conjugation so that a chemical or enzymatic deconjugation step may be required. Often only the use of radiotracers

allows a time- and cost-efficient development of such extraction and the required cleanup methods.

- *Volatile analytes.* As residue analysis is also trace analysis in the lower ppm ($mg\,kg^{-1}$) to ppb ($\mu g\,kg^{-1}$) range, concentration steps usually involve evaporation of solvents (sometimes with traces of water present) to near dryness. The volatility of analytes can be deduced from their elution temperatures in GC, and thus whenever an analyte elutes from a nonpolar GC phase of film thickness $\leq 0.25\ \mu m$ below approximately 150 °C, losses due to co-evaporation during concentration by the rotary evaporator or by a stream of nitrogen need to be avoided.
- *Labile analytes.* Labile analytes may degrade during extraction and cleanup when stress in terms of temperature or pH is applied. They also tend to degrade on GC injection and may even undergo extensive fragmentation during MS ionization even with soft techniques such as ESI or CI. Therefore, one needs to consider derivatization at an early stage of the analytical method, thus enhancing stability and possibly detectability.

3.3 Beyond the limits

Residue analytical chemistry has extended its scope in recent decades from the 'simple' analysis of chlorinated, lipophilic, nonpolar, persistent insecticides – analyzed in the first SiO_2 fraction after the all-destroying sulfuric acid cleanup by a gas chromatography/electron capture detection (GC/ECD) method that was sometimes too sensitive to provide linearity beyond the required final concentration – to the monitoring of polar, even ionic, hydrophilic pesticides with structures giving the chemist no useful feature other than the molecule itself, hopefully to be ionized and fragmented for MS or MS^n detection.

The required limit of quantitation (LOQ) and limit of detection (LOD) have been extended to the parts per billion range as the European Community (EC) 'baby food'-related guideline and the US 'consumer basket' requirements became effective.

Modern analytical techniques in combination with conventional analytical experience and thinking thus try to meet these new requirements by pushing residue analysis to extended limits.

References

1. H. P. Thier and H. Zeumer (eds.), 'DFG Manual of Pesticide Residue Analysis,' VCH, Weinheim, 1987, pp. 71–74, 75–78 and 383–400.
2. W. Specht, S. Pelz, and W. Gilsbach, *Fresenius' J. Anal. Chem.*, **353**, 183 (1995).
3. Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, 'Official Collection of Test Methods According to §35 LMBG (Law of Food and Commodities),' edited Beuth Verlag, Berlin, 1999, Modular Multi Method L 00.00-34.
4. C. D. S. Tomlin (ed.), 'The Pesticide Manual,' twelth edition, Version 2.1, British Crop Protection Council, Farnham, 2001–2.

Best practices in establishing detection and quantification limits for pesticide residues in foods

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1 Introduction

Today, increased globalization has resulted in easy movement of agricultural products from one part of the world to another. The diversity in rules and regulations across the different nations has prompted the global harmonization of different regulatory agencies to establish joint tolerances or maximum allowable residue limits (MRLs), and establish best/uniform risk assessment guidelines. This has led to the necessity to adopt uniform methods to define the limitations of an analytical method. Without an appropriate definition, analyte concentrations reported by a method can be meaningless because calculated values of detection limits can vary over an order of magnitude depending on the method used to determine these values.[1,2]

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The definitions of method detection and quantification limits should be reliable and applicable to a variety of extraction procedures and analytical methods. The issue is of particular importance to the US Environmental Protection Agency (EPA) and also pesticide regulatory and health agencies around the world in risk assessment. The critical question central to risk assessment is assessing the risk posed to a human being from the consumption of foods treated with pesticides, when the amount of the residue present in the food product is reported 'nondetect' (ND) or 'no detectable residues'.

If the analyte of interest is not detected by the instrument (or analytical procedure), does it mean that there is not a single molecule of that analyte present in the food product? Or does it mean that the analyte is present at a concentration that is just below the capability of the instrument? Or is it present at a concentration somewhere between these two extremes?

One major problem caused by Section 409 (c) (3) of the Federal Food, Drug and Cosmetic Act, commonly known as the 'Delaney Clause', which governed the registration of pesticides was the statement, 'No additive shall be deemed safe if it is found to induce cancer when ingested by man or animal, . . . '.[3] Dr Fred R. Shank, Director, Center for Food Safety and Applied Nutrition, US Food and Drug Administration, in

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his statement before the US House of Representatives' subcommittee on Health and the Environment, stated, 'The Delaney Clause read literally, requires absolute safety and would prevent the establishment of any tolerance for a residue in a processed food of any pesticide that is a human or animal carcinogen'.[4] He went on to add that at the time, 'the number of known or postulated carcinogens was fairly small, and the then state-of-the-art capability to detect a substance at a level of a few parts per million was considered ultra-sensitive.' At the time of passage of the Delaney Clause (1958), it was assumed that if residues were not detected then they were absent. As technology improved, more sensitive techniques capable of detecting residues in the sub-parts per trillion became available, thereby exposing the fallacy of this assumption. This clearly highlights the importance of defining the limitations of the analytical method when reporting the results of a test as ND.

Today, when a pesticide with no detectable residues is registered for use, a 'Tolerance' or 'maximum residue limit' (MRL) is established at the lowest concentration level at which the method was validated. However, for risk assessment purposes it would be wrong to use this number in calculating the risk posed to humans by exposure to the pesticide from the consumption of the food product. This would be assuming that the amount of the pesticide present in all food products treated with the pesticide and for which no detectable residues were found is just less than the lowest level of method validation (LLMV). The assumption is wrong, but there is no better way of performing a risk assessment calculation unless the limit of detection (LOD) and limit of quantification (LOQ) of the method were clearly defined in a uniformly acceptable manner.

In 1996, the US Senate and House of Representatives passed the Food Quality Protection Act (FQPA).[5] In order to make their exposure and risk assessments as accurate as possible, the US EPA Office of Pesticide Programs (OPP) has established guidelines to determine a value to assign for NDs.[6] These guidelines can be summarized as follows.

In the absence of any additional evidence implying lower residues:

1. for that percentage of the commodity that was not treated with the pesticide, a value of zero would be assigned;
2. for the remaining fraction, if a valid limit of detection exists, a value equal to half the LOD would be assigned;
3. if an LOD is not properly defined but a valid LOQ exists, a value equal to half the LOQ would be assigned;
4. if neither the LOD nor the LOQ are properly defined, then the value of the LLMV would be assigned;
5. if both the LOD and the LOQ are properly defined, and residues reported are between these two values, then a value equal to half the LOQ would be assigned.

This policy seems to be rational and would be of tremendous help to registrants in getting more uses registered for their products. This would be especially useful when new uses (worth millions of dollars to the registrant) were to be added to the label in cases where no detectable residues were found in the food product and the 'risk cup' (cumulative exposure risk) was nearly full. The following example highlights this point:

Risk cup = 0.2 mg kg^{-1}
LLMV = 0.05 mg kg^{-1}

If no detectable residues were present, the value used for risk assessment purposes would be 0.05 mg kg^{-1}. This would allow only four uses with no detectable residues and even fewer uses where residues were present. If an appropriate LOD and LOQ had been calculated and reported as follows:

LOD = 0.015 mg kg^{-1}
LOQ = 0.045 mg kg^{-1}

As per US EPA guidelines, the value used for risk assessment purposes would now be 0.0075 mg kg^{-1}, thereby permitting up to 26 uses for the product with no detectable residues.

There are several factors involved in defining the limitations of an analytical method. Selecting the right method for defining these limitations can be as important as the actual definitions. Factors that must be taken into consideration in defining detection and quantification limits are:

- instrumental noise
- matrix effects and interferences
- variability in extraction procedures, etc.

Several articles and books have been published dealing with this subject. In this article, some of the techniques which are relevant to methods for the analysis of foods for pesticide residues will be discussed.

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1.1 Definitions

Several terms have been used to define LOD and LOQ. Before we proceed to develop a uniform definition, it would be useful to define each of these terms. The most commonly used terms are limit of detection (LOD) and limit of quantification (LOQ). The 1975 International Union of Pure and Applied Chemistry (IUPAC) definition for LOD can be stated as, '*A number expressed in units of concentration (or amount) that describes the lowest concentration level (or amount) of the element that an analyst can determine to be statistically different from an analytical blank*'.[7] This term, although appearing to be straightforward, is overly simplified. It leaves several questions unanswered, such as, what does the term 'statistically different' mean, and what factors has the analyst considered in defining the 'blank'? Leaving these to the analyst's discretion may result in values varying between analysts to such an extent that the numbers would be meaningless for comparison purposes.

Later in 1995, IUPAC came up with additional recommendations for the definition of LOD.[8] Detection limit is defined as, '*The minimum detectable value of the net signal (or concentration) is that value for which the false negative error is β, given α. "α" is defined as the probability for a false positive ("analyte present" result when that is wrong) and "β" is defined as the probability of a false negative ("analyte absent" result when that is wrong)*'.[8] The values of α and β are defined by the analyst. This definition adds additional parameters to the definition of LOD, but does not solve

the fundamental issue of variability in the calculated value depending on the method used and parameters included in calculating LODs. Therefore, it is important that this term be defined in a manner such that it is easy to compare values reported by different analysts and laboratories. For most modern analytical methods, the LOD may be divided into two components, instrumental detection limit (IDL) and method detection limit (MDL).

In modern times, most analyses are performed on an analytical instrument for, e.g., gas chromatography (GC), high-performance liquid chromatography (HPLC), ultraviolet/visible (UV) or infrared (IR) spectrophotometry, atomic absorption spectrometry, inductively coupled plasma mass spectrometry (ICP-MS), mass spectrometry. Each of these instruments has a limitation on the amount of an analyte that they can detect. This limitation can be expressed as the IDL, which may be defined as the smallest amount of an analyte that can be reliably detected or differentiated from the background on an instrument.

The IDL is dependent on various factors such as sensitivity of the detector for the analyte of interest and electronic and detector (instrumental) noise of various origins, e.g., thermal noise, shot noise, flicker ($1/f$) noise, environmental noise, etc.[8] Several books and articles have been published on the different types of instrumental noise, e.g., Skoog and Leary's 'Principles of Instrumental Analysis'.[9]

As the sensitivity increases, the IDL decreases, and as the instrumental noise decreases, so does the IDL. These aspects are key to selecting the correct instrument/detector system to perform the analysis.

Another factor of interest in defining the instrumental limitations is the instrumental quantification limit (IQL), which may be defined as the smallest amount of an analyte that can be reliably quantified by the instrument.

These two terms (IDL and IQL) define only the limitations of the instrument. When analyzing real-life samples such as plant or animal tissue or even soil and ground water samples, matrix interference must be taken into consideration in order to define detection limits. This is because these 'real-life matrices' are made up of hundreds (or even thousands) of compounds. These compounds may interfere in several ways in the detection and quantification of the analyte of interest.

In some cases, one or more of the matrix components may either elute at the same time as the analyte of interest in GC or HPLC or absorb or emit in the same wavelength range as the analyte. In other cases, the matrix components may either enhance or quench the analyte signal. In some cases, the matrix components catalyze reactions involving the analyte, for example, in GC or HPLC analysis involving pre- or post-column derivatization, matrix components may enhance or decrease the formation of the derivatized adduct. This may also be true of UV/VIS and fluorescence spectroscopy involving chemical modification of the analyte to enhance detection. As far as possible, the extraction method should be able to remove most of the interfering components. However, in complex matrices complete elimination of interfering components is impossible and therefore these effects must be taken into account when determining the LOD for an analyte–matrix combination. This leads us to a second set of terms that must be defined.

The method detection limit (MDL) is a term that should be applied to extraction and analysis methods developed for the analysis of specific analytes within a matrix. The MDL can be defined as the smallest amount of an analyte that can be reliably

detected or differentiated from the background for a particular matrix (by a specific method). All matrix interference must be taken into consideration when determining the MDL. Similarly, the method quantification limit (MQL) can be defined as the smallest amount of an analyte that can be reliably quantified with a certain degree of reliability within a particular matrix (by a specific method).

Finally, it is important to define the lowest level of method validation (LLMV). The LLMV is defined as the lowest concentration level expressed in terms of amount of analyte in the matrix, at which the method (extraction/analysis procedure) was validated or proven to be capable of reliably quantifying.

Depending on the method used to define the detection limit, the value can vary up to an order of magnitude, thereby rendering these numbers meaningless. This is why it is very important to have uniform definitions for each of these terms, in order to be able to compare values across matrices, methods and laboratories.[1]

2 Methods for defining LOD and LOQ

2.1 IUPAC method

In 1975, the IUPAC defined the LOD in terms of concentration (c_L) and the signal (x_L) generated by a solution of concentration c_L.[1] They defined the value of x_L in terms of the mean blank signal ($\bar{x}_B$) and the standard deviation (s_B) of these blank measurements as

$$x_L = \bar{x}_B + k \cdot s_B \tag{1}$$

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where k is a numerical factor chosen in accordance with the confidence level desired .[1] Long and Winefordner[1] further link c_L to x_L as follows:

$$c_L = \frac{(x_L - \bar{x}_B)}{m} \tag{2}$$

where m is defined as 'analytical sensitivity' and expressed as the slope of the calibration curve line obtained from the linear regression analysis.[1] By substituting the value of x_L from equation (1) into equation (2), Long and Winefordner define c_L as

$$c_L = \frac{(k \cdot s_B)}{m} \tag{3}$$

Long and Winefordner along with several other authors agree on a value of $k = 3$, which allows a confidence level of 99.86% if the values of x_B follow a normal distribution, and 89% if the values of x_B do not follow a normal distribution.[1,10] A value of $k = 2$ has also been used by some workers, but this decreases the confidence level in c_L. The definition of LOD was later expanded on by IUPAC in 1995 to include the probabilities of false positives and negatives.

Both IUPAC and the American Chemical Society (ACS) have accepted the definition of c_L shown in equation (3). However, there are a few problems associated with

using this definition in today's automated chromatographic systems used for pesticide residue analysis in food/soil and also pesticide/drug analysis in animal fluids and tissues.

Modern chromatographic equipment is fitted with computers which gather data from the detector, process the data into a chromatogram (detector response vs time), integrate areas under peaks or measure peak heights and present the peak area/height in a report (printout or screen display). These integrators offer several modifiable parameters which must be set by the analyst in order to consistently integrate the peak area or measure peak height for standards, controls, fortified controls, and treated samples. Integration parameters cannot be changed within a batch or sequence.

Some of the parameters involved are as follows:

1. *Integrator on and off times*: Lets the computer know the time range of interest within the chromatogram [elution time range of the analyte(s) of interest].
2. *Minimum peak height/area*: Lets the computer know the minimum peak height or area below which the computer may disregard the peak (eliminates noise).
3. *Peak width definition (threshold)*: Lets the computer know how to distinguish between true peaks and detector spikes.
4. *Manual integration*: Permits the analyst to integrate the peak(s) of interest manually. In this case the analyst should be consistent in defining the baseline.

Figure 1 shows a chromatogram that may be obtained from the injection of a plant/animal tissue extract containing the analyte of interest (peak 11) after normal cleanup procedures. Figure 2 shows a chromatogram of the analyte standard in a pure solution and Figure 3 shows a chromatogram of an untreated control sample extract. If the integration parameters and ranges were improperly set, or set so as to integrate even the smallest 'blips', then all the peaks 1–12 and also the instrumental fluctuation observed along the baseline (peak 13) would be integrated. This would

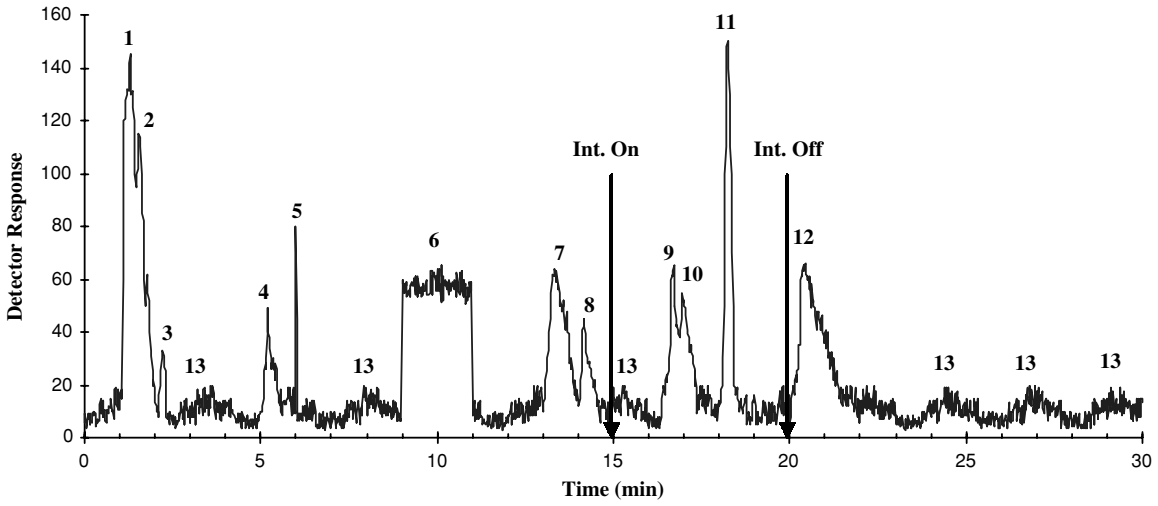


Figure 1 Example of a sample chromatogram with the analyte peak (11) eluting at 18.23 min, solvent peaks (1–3), matrix component peaks (4, 7–10, 12), and instrumental noise (5, 6, 13)

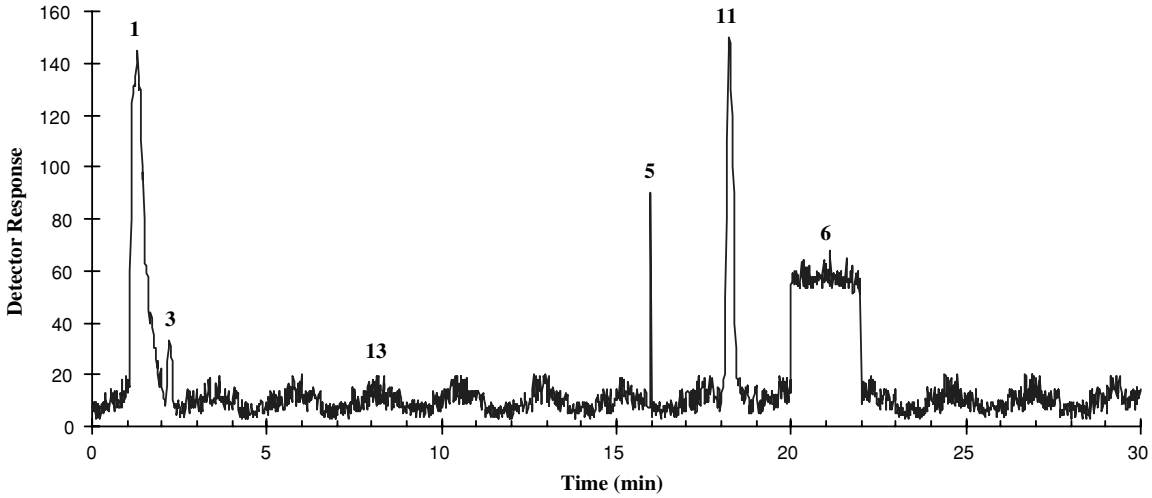


Figure 2 Example of a standard chromatogram (in pure solvent without matrix components) with the analyte peak (11) eluting at 18.23 min, solvent peaks (1, 3), and instrumental noise (5, 6, 13)

take a long time to generate and would overwhelm the analyst with a lot of useless information.

If the threshold and minimum peak heights/areas were appropriately defined, then only peaks 1–4 and 7–12 would be integrated, making the report much easier to read. Peaks 5, 6 and 13 are due to instrumental/detector noise. This would still provide a lot of extraneous information; however, the instrumental noise has been eliminated.

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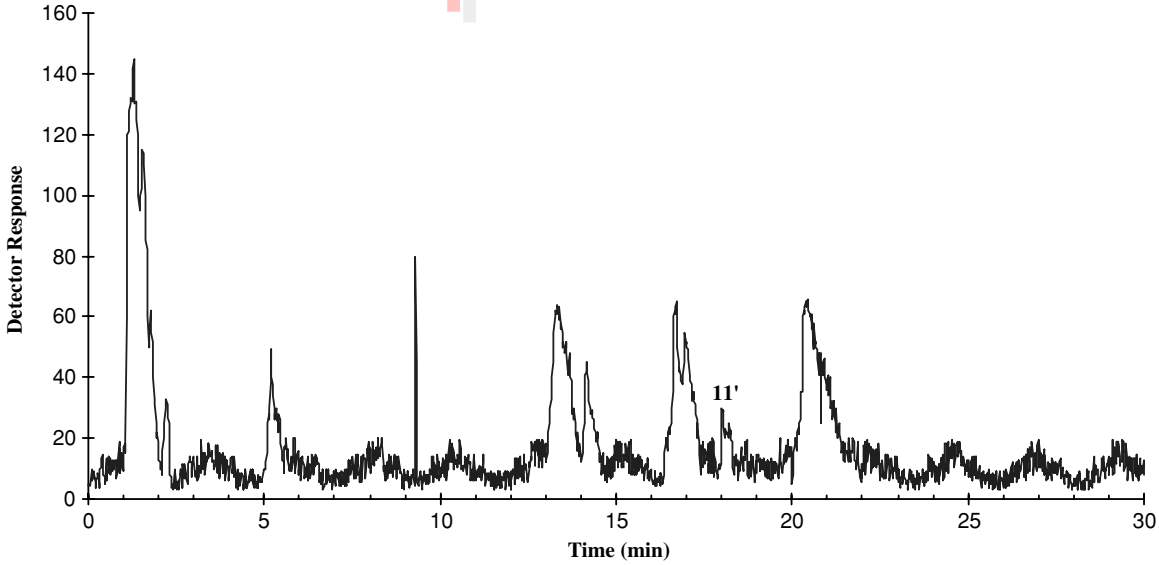


Figure 3 Example of an untreated control chromatogram with the interfering peak (11) eluting at 18 min, solvent peaks (1–3), matrix component peaks (4, 7–10, 12), and instrumental noise (5, 13)

Additionally, setting the correct 'integrator on and off' times would eliminate peaks 1–8 and 12, which are not of interest since the retention time of the analyte has been determined from the injection of a standard (Figure 2). The 'integrator on and off' times also need to be carefully determined.

Each of these parameters needs to be carefully set. Setting too large a range would provide the analyst with a lot of extraneous information, and setting too small a range would result in the possibility of incomplete integration due to slight shifts in analyte retention times. Also, setting the threshold or minimum peak height/area too high may result in the computer ignoring peaks of interest if they fall below the set minimum.

From the above discussion, it becomes apparent that if the standard deviation of the blank (s_B) had to be determined, the integration parameters would have to be set so as to integrate the background around the peak of interest. This would lead to a lot of useless information being generated and also improper integration of the analyte peak. The analyst would then be challenged to distinguish between the noise affecting the analyte peak and that which does not. Determining the value of s_B by integrating over a very narrow range (the width of the peak) may exclude matrix interferences (peaks eluting close to the analyte), which could result in under-calculating the value of s_B. On the other hand, integrating over too wide a range may result in noninterfering peaks contributing to the value of s_B. This makes the determination of the value of s_B very subjective, a major drawback in using the IUPAC method for calculating the MDL in dynamic systems such as chromatography. However, the IUPAC method provides a good estimate of MDL for techniques using static measurements such as spectrophotometers (UV/VIS, fluorimeters, AAS, ICP-MS, etc.).

2.2 *Propagation of errors method*

A variation of the IUPAC method called the 'propagation of errors' (PE) method has been discussed by Long and Winefordner.[1] In the PE method, the LOD is defined as

$$c_L = \frac{k \cdot \left\{ s_B^2 + s_i^2 + \left[\left(\frac{i}{m} \right)^2 \cdot s_m^2 \right] \right\}^{1/2}}{m} \tag{4}$$

where i is the intercept, s_i is the standard deviation of the intercept and s_m is the standard deviation of the slope m. If the value of s_m is small, then equation (4) reduces to

$$c_L = \frac{k \cdot \left(s_B^2 + s_i^2 \right)^{1/2}}{m} \tag{5}$$

If the value of s_i is much less than that of s_B, then equation (5) reduces to equation (3), the IUPAC definition.

It is important to note that the matrix effects, interferences, and variability in method efficiency are to be factored in when determining the MDL. If this was not done then only the background noise (see Figure 2, peak 13) would be considered in the definition of the MDL. In real-life samples there is a good possibility that matrix component peaks would either co-elute or elute at retention times close to

the analyte peak, thereby affecting the analyte signal (see Figure 3, peak 11). In order to factor these interferences into the determination of MDL, the calibration curves would have to be prepared by fortifying control samples with the analyte of interest at different concentrations around an estimated detection limit (within one order of magnitude). The fortified samples would then be extracted and analyzed and a calibration curve prepared in this manner. Furthermore, in order to measure s_i and s_m accurately, data from a minimum of five calibration curves would have to be evaluated. This would make the procedure tedious and time consuming for dynamic systems such as chromatography.

On the other hand, for static systems such as UV/VIS spectrophotometry, AAS, ICP-MS, etc., the PE method would be very easy to use since the values of s_B and s_i could be easily determined from analyzing multiple untreated control extracts. Furthermore, the PE method would be preferred over the IUPAC method because errors in analyte measurements can be incorporated into the MDL.[1]

2.3 *Hubaux–Vos approach*

A method for defining detection limits was discussed by Hubaux and Vos in a paper published in 1970.[11] This method involves the generation of multiple calibration curves and factors in the variability in the slope and intercept of the calibration curve. The Hubaux–Vos approach also factors analyst desired probabilities for false positives and negatives into the determination of the detection limit. Although these are important factors that must be factored in for certain types of analyses, the Hubaux–Vos approach is complicated and becomes very tedious and time consuming when matrix effects and interferences are to be factored in for determining the MDL. The same steps as outlined earlier, namely preparing the calibration curves from fortified matrix samples, would have to be applied when using this approach to determine the MDL. Additional information regarding the Hubaux–Vos approach can be found in the original paper.[11]

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2.4 *Two-step approach (proposed by the US EPA)*

A method for determining the LOD and LOQ for water samples was proposed by the US EPA.[12] This method has also been discussed by Roy-Keith Smith in his book titled 'Handbook of Environmental Analysis'.[13] The method has also been proposed by the US EPA in their guidelines for 'Assigning Values to Non-detected/Non-quantified Pesticide Residues in Human Health Food Exposure Assessments'.[6]

This approach involves two steps for the determination of the MDL and MQL:

1. determining the instrumental limit of detection (IDL) and quantification (IQL) and using these values to estimate the MDL and MQL;
2. calculating the MDL and MQL for the extraction/analysis method for the crop/chemical combination.

For this approach, it is very important that both steps be followed in order that the calculated values of MDL and MQL are reliable. If only the first step is applied, then

the calculated value would not take matrix interferences and effects into consideration. On the other hand, if only the second step is used, the concentration of the fortified control samples used in calculating the standard deviation would bias the values of MDL and MQL. There are several ways in which the IDL and IQL could be determined. Any of the above-mentioned approaches can be used.

The simplest method for estimating the MDL and MQL would be to measure the peak-to-peak noise ($N_{p–p}$) around the analyte retention time and then estimate the concentration (of the analyte in the matrix) that would yield a signal equal to three times the $N_{p–p}$ (estimated MDL).

Alternatively, the following method would provide the analyst with a more reliable estimate of MDL:

1. analyze several (at least five) untreated control extracts;
2. measure the $N_{p–p}$ for each of the extracts;
3. calculate the average $N_{p–p}$ for the measurements;
4. calculate the concentration of a solution that would produce a signal three times the $N_{p–p}$ (taking into consideration expected losses of analyte during extraction/cleanup steps);
5. estimate the value of MDL by calculating the amount of analyte in the matrix that would yield the signal (using concentration/dilution factors).

2.5 RMSE method

Another method recommended by the US EPA[6] for estimating the LOD involves the generation of a calibration curve and calculating the root mean square error (RMSE). This method should be applied when a linear relationship exists between detector response and analyte concentration. The RMSE method involves the following steps:

1. Generate a 4–5-point calibration curve with standards of concentrations within an order of magnitude of the estimated detection limit. For this purpose, the detection limit may be estimated as a concentration that would yield a signal three times $N_{p–p}$. The calibration curve should be generated by plotting detector response (x) vs concentration (c).
2. Perform a regression analysis on the calibration curve and calculate the values of slope (m), intercept (i) and r^2 for a number of standards n.
3. The calibration curve can be defined by the following equation:

$$x = m \cdot c + i \tag{6}$$

4. Based on the values of slope m and intercept i, calculate the predicted response (x_p) for each of the standards.
5. Calculate the error (E) associated with each measurement $|x_p - x|$.
6. Calculate the square of the errors for each standard and then calculate the sum of the square of the errors ($\sum E^2$) for a number of points n.

7. The RMSE is then calculated as follows:

$$\text{RMSE} = \left[\frac{\sum_{j=1}^{n} E_j^2}{n-2} \right]^{1/2} \tag{7}$$

8. The predicted response at the IDL (x_L) is calculated as follows:

$$x_L = i + (3 \cdot \text{RMSE}) \tag{8}$$

9. Rearranging equation (6), the IDL (c_L) can be calculated as

$$c_L = \frac{(x_L - i)}{m} \tag{9}$$

Combining equations (8) and (9), we obtain

$$c_L = \frac{3 \cdot \text{RMSE}}{m} \tag{10}$$

The value of c_L determined here is measured in terms of concentration and solution. This value does not take matrix interferences into account since RMSE was determined from calibration standards. Therefore, this value should be reported as the IDL. This value provides a good starting point for the next step, which is calculating the MDL.

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Comparing equations (10) and (5), the IUPAC definition for detection limit, the difference is that RMSE is used instead of s_B. For dynamic systems, such as chromatography with autointegration systems, RMSE is easier to measure and more reliable than s_B for reasons discussed earlier. Both are measures of variance and, although dissimilar, provide similar information. This is apparent in the equations used to calculate the values of s_B and RMSE:

$$s_B = \frac{\sum_{j=1}^{n} \left(x_{j_B} - \bar{x}_B \right)}{n-1} \tag{11}$$

and

$$\text{RMSE} = \left[\frac{\sum_{j=1}^{n} \left(x_j - x_{j_p} \right)^2}{n-2} \right]^{1/2} \tag{12}$$

The LOQ is regarded as the lower limit for precise quantitative measurements.[2] Several authors, such as Miller and Miller,[2] Skoog and Leary,[9] and Smith,[13] have suggested that the LOQ be defined as a concentration which would produce a signal 10–12 times s_B. At this point, the standard deviation is small enough (approximately 10–15%) so that the quantitated value can be deemed reliable.

Extending this principal to equation (10), the instrumental quantification limit (IQL, c_q) may be calculated using the equation

$$c_q = \frac{10 \cdot \text{RMSE}}{m} \tag{13}$$

2.6 The $t_{99}s_{LLMV}$ method

The second step in the two-step approach involves calculating the values of MDL and MQL. This method has been described previously by the US EPA[12] and by Smith[13] for the analysis of water samples. This method involves the following steps:

1. Weigh seven or more sub-samples (ground and homogenized) of an untreated control sample of the matrix of interest. The weight of each sub-sample should be the same as that proposed to be used during sample analysis.
2. Estimate the LOQ using any of the methods described earlier and equate the value in terms of amount in the matrix, factoring in any concentration and dilution factors from the extraction procedure.
3. Fortify each of these sub-samples with the analyte(s) of interest such that the concentration of the analyte(s) in the matrix equals the estimated limit of quantification (ELOQ).
4. Extract each of these fortified control samples using the extraction procedure used for the sample analysis.
5. Analyze each of the final extracts using the method used for sample analysis.
6. Determine the amount of residue found in each of the fortified samples.
7. Calculate the standard deviation of these measurements (s_{ELOQ}).
8. Determine the 'one-tailed t-statistic' for $n - 1$ observations at the 99% confidence level [$t_{99_{(n-1)}}$].
9. The MDL for the matrix/analyte(s) combination and the extraction/analysis procedure is defined as

$$\text{MDL} = t_{99_{(n-1)}} \cdot s_{\text{ELOQ}} \tag{14}$$

For seven replicates (six degrees of freedom) of the fortified control samples,

$$t_{99_{(n-1)}} = 3.143$$

As discussed earlier, a concentration that would produce a signal of approximately 10–12 times the standard deviation of the blank (or in this case s_{ELOQ}) is considered to be the limit of quantification. Therefore, if the LOQ was set at 10 times s_{ELOQ}, for 7 replicates (6 degrees of freedom) of the fortified control samples

$$t_{99_{n-1}} = 3.143$$
$$10/t_{99_{n-1}} = 10/3.143 = 3.182, \text{ which can be rounded off to } 3$$

then the method quantitation limit (MQL) for the matrix/analyte(s) combination and

the extraction/analysis procedure is defined as

$$\text{MQL} = 3 \cdot \text{MDL} \tag{15}$$

This would provide a concentration limit above which fairly precise quantitative measurements can be reported.

Alternatively, during the course of method validation and sample analysis, control samples fortified at the ELOQ (determined by one of the methods described above) are extracted and analyzed. The standard deviation of these fortified control samples (s_{LLMV}) can also be used to calculate the MDL and the MQL for the method. In the latter case, s_{LLMV} would replace s_{ELOQ} in equation (14).

The value of MDL and MQL calculated by the two-step process described above takes into consideration several factors which affect the analyte signal, including:

- instrumental noise
- variability in instrument sensitivity
- variability in method efficiency
- matrix effects and interference

However, it is extremely important that the estimated LOD and LOQ be accurately determined. The fortification concentration greatly influences the final value of MDL and MQL determined by this method. If too high or too low a value of ELOQ is chosen for fortification for determining the MDL, then the calculated MDL and MQL may be different from the actual method capability.

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The US EPA recommends that if the calculated values of LOQ (MQL) are significantly different from the estimated values, then steps 1–7 above should be repeated with the new 'estimates' of the LOD/LOQ and the MDL and MQL should be recalculated.[12] This should be done till the calculated values of LOD and LOQ are in the 'range' of the estimated values (LLMV = 2–5 times the MDL).

Although time consuming, this is important for determining accurate values of MDL and MQL. However, if the ELOQ/LLMV is properly determined by any of the methods described above, then an experienced chemist should be able to determine accurately the fortification levels (LLMV) for calculating MDL and MQL, thereby avoiding time-consuming repetitions. The two-step approach is a fairly accurate way for determining method limitations.

3 Confirmation

Once the MDL has been calculated, it is important to prove that the chosen analytical procedure is practically capable of detecting the analyte(s) at the MDL. To prove the practicality of the MDL, the analyst should spike triplicate sub-samples of an untreated control sample at the MDL, extract the fortified control samples and analyze them on the instrument. Well defined chromatographic peaks would prove the validity of the calculated MDL.

Table 1 Representative data: evaluating data obtained from various studies for calculating the LOD and LOQ values for the extraction/analysis procedure using the '3(RMSE)/slope' method to estimate the LOD/LOQ and the '$t_{99_{(n-1)}} s_{ELOQ}$' method to calculate the MDL and MQL

Method[a]	Pesticide	Matrix	ELOQ (mg kg^{-1})	LLMV (mg kg^{-1})	Av. recovery ± standard deviation	Calc. MDL (mg kg^{-1})	Calc. MQL (mg kg^{-1})	Ref.
GC/ECD	Fipronil	Onion	0.002	0.005	0.0046 ± 0.0004	0.001	0.003	15
GC/ECD	Clopyralid	Canola	0.065	0.1	0.1124 ± 0.016	0.04	0.12	16
GC/ELCD	Sulfentrazone	Cabbage	0.03	0.05	0.0503 ± 0.005	0.014	0.042	17
GC/NPD	Zn_3P_2	Cucumber	0.05	0.05	0.0351 ± 0.0048	0.014	0.042	18
GC/FPD	Dimethoate	Grass	0.011	0.02	0.0196 ± 0.003	0.0086	0.026	19
GC/MS	Clopyralid	Canola	0.035	0.05	0.0593 ± 0.004	0.011	0.034	20
GC/MS	Quinoxyfen	Cantaloupe	0.005	0.01	0.0096 ± 0.00085	0.0027	0.008	21
LC/UV	Desmedipham	Spinach	0.024	0.05	0.0359 ± 0.002	0.0074	0.022	22
LC/MS	Fenhexamid	Pepper	0.031	0.02	0.0175 ± 0.0024	0.0065	0.002	23

[a] GC/ECD, gas chromatography/electron capture detection; GC/ELCD, gas chromatography/electrochemical detection; GC/NPD, gas chromatography/nitrogen–phosphorus detection; GC/FPD, gas chromatography/flame photometric detection; GC/MS, gas chromatography/mass spectrometry; LC/UV, liquid chromatography/ultraviolet detection; LC/MS; liquid chromatography/mass spectrometry.

3.1 Representative data

The Interregional Research Project No. 4 (IR-4) was formed in the USA in 1963 to address the problem of lack of available pest control products for minor food crops (grown on 300 000 acres or less).[14] The problem of the lack of appropriate methods to define method limitations has often plagued IR-4 analytical laboratories and chemists who have several years of experience in method development for pesticide residues in food. When the US FQPA was passed in 1996, the issue of calculating cumulative risks due to exposure from all sources has placed a very high burden on minor crops. The smaller market of minor crops has led some registrants to drop the use of pesticides in minor crops in order to retain the more profitable major crop uses such as corn, cotton, wheat and soybeans. For pesticide uses in minor crops where the use results in no detectable residues, IR-4 chemists have been challenged to develop methods with extremely low detection limits and prove the lack of detectable residue at these low limits, thereby increasing the number of minor uses by 'freeing up space in the use cup'.

Data from several laboratories within the Interregional Research Project No. 4 (IR-4) in the USA have been evaluated for determining the values of MDL and MQL. These data have been presented in Table 1. The two-step procedure described in the EPA guideline[6] was used to calculate the values of MDL and MQL. For the first step, the slope, intercept and RMSE values for the first three calibration curves of each study were separately calculated, then the IDL and IQL values calculated and the value of LOQ estimated for the method. These values were compared with the actual values of LLMV. The standard deviation of the spike recoveries at the LLMV (s_{LLMV}) was used to calculate the MDL and MQL. The values of LLMV were separately determined by the laboratory not using any of the methods described in this article.

Evaluating the data presented in Table 1 indicates that the values of ELOQ, LLMV and MQL are comparable, implying that the calculated values of MDL are fairly

Table 2 Comparison of methods for calculating detection and quantification limits for analytical methods used for food analysis

Method	Simple and easy to apply	Considers variability of calibration curve	Considers method efficiency and matrix effects	Variability between laboratories and analysts	Good for estimating LOD/LOQ	Comments
N_{p-p}	Yes	No	Yes	High	Yes	Very dependent on analyst interpretation
k_{S_B}/m	No	No	No	Moderate	No	Difficult to implement
PE	No	Yes	No	Low	Acceptable	Difficult to implement
Hubaux–Vos	No	Yes	No	Low	Very tedious and time consuming	Impractical for complex matrices
RMSE	Yes	Yes	No	Low	Yes	Good for IDL but not MDL
$t_{99}s_{LLMV}$	Yes	Yes	Yes	Low	No	Very dependent on value of LLMV chosen
Two-step approach using $t_{99}s_{LLMV}$ method	Yes	Yes	Yes	Low	Yes	Best for calculating MDL and MQL

reliable. In a few cases (not reported here), the value of MQL was significantly (more than three times) different from the LLMV. In cases such as these, it would be advisable to repeat the procedure as discussed earlier.

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4 Conclusions

Several methods have been discussed for the determination of method limitations when evaluating procedures for the determination of pesticides in food. A brief comparison of the methods discussed for the determination of the detection and quantification limits of methods used for the analysis of food products can be found in Table 2.

Although accepted by IUPAC and ACS, the '$k \cdot s_B/m$' definition is hard to implement and does not take either variability in method efficiency or matrix effects into consideration. This would be rectifiable if the calibration curves were prepared from control matrix samples fortified at different concentrations (within one order of magnitude of an estimated LOD).

A better alternative would be to use the 'propagation of errors' definition, which takes into consideration values of both s_B and s_i when calculating the MDL. This would involve generating at least five calibration curves in order to obtain an accurate measurement of s_i and s_m.

The $3N_{p-p}$ approach, although simple, leaves too much to the analyst's discretion, thereby rendering the values obtained hard to compare between analysts and laboratories. This method may be used in estimating the LOD and LOQ in the two-step approach.

The two-step approach involving the RMSE (or the $3 \cdot N_{p-p}$) method for estimating the LOQ and $t_{99_{(n-1)}} \cdot s_{ELOQ}$ is the most practical method for determining the MDL and MQL of the extraction/analysis procedure because it incorporates matrix effects and interferences and also variability of method efficiency in the final calculation. The $t_{99_{(n-1)}} \cdot s_{ELOQ}$ method can be applied to data generated during the course of a pesticide registration study. When data obtained during the course of a study are used, the standard deviation of the LLMV spike recoveries (s_{LLMV}) can be used instead of s_{ELOQ} provided that the LLMV was appropriately determined by one of the methods discussed in this article or any other statistically valid method.

Since several methods appear to be acceptable, it is important that when reporting values for MDL and MQL, the method used to define these values be clearly identified.

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References

1. G.L. Long and J.D. Winefordner, *Anal. Chem.,* **55**, 713A (1983).
2. J.C. Miller and J.N. Miller, 'Statistics for Analytical Chemistry,' Ellis Horwood, Chichester (1988).
3. US Food and Drug Administration, 'Federal Food, Drug and Cosmetic Act (Amended 1997).' Also available on the World Wide Web: **http://www.fda.gov/opacom/laws/fdcact/fdctoc.htm**.
4. F.R. Shank, Statement Before the Subcommittee on Health and the Environment, Committee on Energy and Commerce, US House of Representatives (June 19, 1991). Also available on the World Wide Web: **http://www.fda.gov/bbs/topics/SPEECH/SPE00002.htm**.
5. 'Food Quality Protection Act of 1996, Public Law 104–170, 104th US Congress (August 3, 1996). Also available on the World Wide Web: **http://frwebgate.access.gpo.gov/cgi-bin/getdoc.cgi?dbname=104_cong_bills&docid=f:h1627enr.txt.pdf**.
6. US EPA, 'Assigning Values to Non-detected/Non-quantified Pesticide Residues in Human Health Food Exposure Assessments,' Guidance Document: Office of Pesticide Programs, US Environmental Protection Agency, Washington, DC (March 23, 2000). Also available on the World Wide Web: **http://www.epa.gov/pesticides/trac/science/trac3b012.pdf**.
7. IUPAC, 'Nomenclature, symbols, units and their usage in spectrochemical analysis–II,' *Spectrochim. Acta, Part B,* **33**, 242 (1978).
8. L.A. Currie, *Pure Appl. Chem.,* **67**, 1699 (1995).
9. D.A. Skoog and J.J. Leary, 'Principles of Instrumental Analysis,' Saunders College Publishing, New York (1992).
10. H. Kaiser, *Anal. Chem.,* **42**, 26A (1970).
11. A. Hubaux and G. Vos, *Anal. Chem.,* **42**, 849, (1970).

12. US EPA, 'Title 40 of the US Code of Federal Regulations, Part 136, Appendix B,' Office of the Federal Register National Archives and Records Administration, Washington, DC (July 1, 1993).
13. R.-K. Smith, 'Handbook of Environmental Analysis,' Genium, New York (1999).
14. G.M. Markle, 'Minor Use Pesticides–Registration (IR-4 Program) Dynamic Project,' in "Encyclopedia of Agrochemicals," Wiley, Chichester, in press.
15. I.-M. Larsson-Kovach, R. Fish, and J. Corley, 'Fipronil: Magnitude of the Residue on Onion (Dry Bulb),' unpublished Analytical Summary Report for IR-4 Study No. 07040, Cornell University, Geneva, NY (2001).
16. J. Corley, B. Glazier, and G. Möller, 'Clopyralid: Magnitude of the Residue on Canola (Rapeseed),' unpublished IR-4 Submission to the US EPA, Rutgers, The State University of New Jersey, North Brunswick, NJ (2001).
17. J. Corley, J. Yoh, and C.C. Turlington, 'Sulfentrazone: Magnitude of the Residue on Cabbage,' unpublished IR-4 Submission to the US EPA, Rutgers, The State University of New Jersey, North Brunswick, NJ (2001).
18. J. Corley, G. Möller, and K. Samoil, 'Zinc Phosphide: Magnitude of the Residue on Cucumber,' unpublished Analytical Summary Report for IR-4 Study No. 04333, University of Idaho, Moscow, ID (1997).
19. K. Samoil, J. Corley, and G. Möller, 'Dimethoate: Magnitude of the Residue on Grass,' unpublished IR-4 Submission to the US EPA, Rutgers, The State University of New Jersey, North Brunswick, NJ (1997).
20. J. Corley, B. Schaffman, T. Taruscio, and G. Möller, 'Clopyralid: Magnitude of the Residue on Canola,' unpublished IR-4 Submission to the US EPA, Rutgers, The State University of New Jersey, North Brunswick, NJ (2001).
21. B. Hung, M. Hengel, and J. Corley, 'Quinoxyfen: Magnitude of the Residue on Cantaloupe,' unpublished data from ongoing IR-4 Study No. 07252, University of California, Davis, CA (2002).
22. I.-M. Larsson-Kovach, W. Kean, R. Fish, and J. Corley, 'Desmedapham: Magnitude of the Residue on Spinach,' unpublished Analytical Summary Report for IR-4 Study No. 01922, Cornell University, Geneva, NY (2001).
23. I.-M. Larsson-Kovach, M. Hummiston, and J. Corley, 'Fenhexamid: Magnitude of the Residue on Pepper (Bell and Non-Bell),' unpublished data from ongoing IR-4 Study No. 07264, Cornell University, Geneva, NY (2002).

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The process of development and validation of animal drug residue methods for US Food and Drug Administration regulatory use

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1 Introduction

The US Food and Drug Administration (FDA) evaluates methods to be used in government regulatory laboratories for the determination and confirmation of drug residues in food derived from animal products. The FDA Center for Veterinary Medicine (CVM) oversees the validation (i.e., demonstration that the method is suitable for use) via a protocol known as a method trial. CVM ensures that the appropriate government laboratories have the tools needed to monitor the Nation's food supply.

In 1906, the original Food and Drugs Act was passed by Congress to prohibit interstate commerce in misbranded and adulterated foods and drugs. The use of poisonous preservatives and dyes in foods and cure-all claims for worthless and dangerous patent medicines were major problems leading to the enactment of this law. The Federal Food, Drug, and Cosmetic (FDC) Act of 1938 extended Federal authority over cosmetics and therapeutic devices. The FDC Act also required that new drugs be shown to be safe before marketing and provided for the establishment of tolerances for unavoidable poisonous substances.

In 1958, the Food Additives Amendment was enacted, requiring manufacturers of new food additives to establish safety and provide a description of practicable methods for determining the quantity of such additive in or on food, and any substance formed in or on food, because of its use. The Delaney Clause prohibited the approval of any food additive shown to induce cancer in humans or animals. The Kefauver–Harris Drug Amendments were passed in 1962 to ensure drug efficacy and greater drug safety. Drug manufacturers were, for the first time, required to prove to the FDA the effectiveness of their products before marketing them. The 1962 law also exempted from the Delaney Clause animal drugs and animal feed additives that induce cancer

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when ingested by man or animal but for which no residue of such drug will be found. This is also known as the Diethylstilbestrol (DES) Proviso.

According to the DES Proviso, a carcinogenic animal drug, feed additive, or color additive could be approved provided that, under the conditions of use specified in proposed labeling and reasonably certain to be followed in practice, no residue of such [substance] will be found (by methods of examination prescribed or approved by the Secretary by regulations), in any edible portion of such animals after slaughter or in any food yielded by or derived from the living animals [see FDC Act, Sections 409 (c)(3)(A)(ii), 512 (d)(1)(I)(ii), and 721 (b)(5)(B)(ii)]. Under 21 CFR 500.84 (c)(2), no residue of a carcinogenic compound is operationally defined. Using a bioassay with an animal model, the maximum lifetime risk of cancer to the test animal of one in one million is determined. The conditions of use of the compound, including any required preslaughter or milk discard time, are set to ensure that the concentration of the residue of carcinogenic concern in the total diet of people does not exceed a concentration calculated to correspond to the one in one million risk of cancer in the animal model. As total diet is not derived from food-producing animals, the FDA adjusts the concentration to account for food intake.

The 1968 Animal Drug Amendments consolidated the regulation of new animal drugs in one section of the FDC Act, Section 512. Under Section 512 (b)(1), any person may file with the Secretary an application with respect to any intended use or uses of a new animal drug. Such a person shall submit to the Secretary as a part of the application . . . (G) a description of practicable methods for determining the quantity, if any, of such drug in or on food, and any substance formed in or on food, because of its use. Sections 409 and 721 of the Act, addressing food additives and color additives, respectively, contain similar language. Although Sections 409, 512, and 721 of the Act and their implementing regulation vary slightly in wording, they have a common purpose – ensuring the safety of residues that people will consume from tissues of treated animals. Therefore, the FDA believes that the same testing requirements should apply to a new animal drug used in, or a food additive or color additive fed to, a food-producing animal.

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The FDA requires [FDC Act, Section 512 (b)(1)(G)] that methods used for the detection and confirmation of drug residues in animal products be practicable. Overseeing the reliability of these methods is the responsibility of the FDA CVM. The methods are corroborated using an interlaboratory evaluation of the method known as a method trial. The method trial is used to demonstrate that the method is suitable for use to detect and confirm drug residues and can be performed by a trained analytical chemist.

Generally a new animal drug is considered to be unsafe and edible animal products containing residues of the drug are considered adulterated unless an approved New Animal Drug Application (NADA) is in effect. Before approving a carcinogenic or noncarcinogenic compound for use in food-producing animals, CVM asks that the sponsor provide an acceptable analytical method (either chemical or biological) capable of reliably measuring the drug residue of concern to ensure that the total residue of toxicological concern is not exceeded. Methods to detect and confirm drug residues are reviewed as part of the application process for food animal drugs and feed additives. Methods that are developed and validated as part of a NADA are referred to as NADA methods, and the process of validating these methods is referred to as

the NADA method trial. This process is more fully explained in Section V of FDA Guideline for Approval No. 3.[1]

The NADA method approval process consists of three phases: (1) method development by the sponsor and generation of information to establish that the method satisfies acceptability criteria; (2) FDA review of the sponsor's data to determine suitability of the method; and (3) the 'method trial', an inter-laboratory study, which determine whether the method meets performance criteria when used in multiple laboratories. The inter-laboratory method trial procedure provides an indication of a method's ability to be used as a practicable and reliable regulatory tool. Sponsors are urged to develop methods that are rugged and exceed rather than meet the minimal standards of acceptability. Those methods that appear marginally acceptable after review often do not pass the inter-laboratory method trial.

NADA methods should be capable of reliably measuring an analyte (i.e., the marker residue) that has a defined quantitative relationship to the total residues of toxicological concern in the tissues of interest, namely the target tissue and muscle. The target tissue is generally the last tissue in which total residues deplete to the permitted maximum safe concentration. When the marker residue is at the tolerance, a defined unique concentration, the total residues have depleted to the respectively established safe concentrations in the target tissue and muscle.

The FDA often develops methods to detect drug residues to support other regulatory needs. In certain instances, new animal drugs may be used legally for unapproved uses, i.e., extra-label use. For example, an approved new animal drug may be used to treat a disease in a species other than the approved species. In addition, animal drugs are sometimes used in an illegal manner in food animals (e.g., clenbuterol or phenylbutazone in food-producing animals). The FDA may need to develop a method to detect unsafe levels of drugs resulting from extra-label or illegal uses. Furthermore, methods may become obsolete with the rapid changes in technology. For many species, the number of approved drugs is very limited. For example, only five drugs are approved for use in aquaculture. Drug residue methods developed for use by the FDA outside of the drug approval process are known as non-NADA methods. As with sponsor-developed methods, FDA developed methods require validation. These FDA methods are validated using a process know as the non-NADA method trial.

The non-NADA method trial process mirrors the NADA process. Methods are developed, reviewed for scientific and technical soundness, and validated in multiple laboratories, and the data generated are analyzed to determine if the method is suitable for its intended use.

2 The method

A method should be able both to quantify the amount of marker drug residue present in the sample and to identify the compound unambiguously. Historically, this required two distinct procedures: a determinative procedure used to quantify the analyte, and a confirmatory procedure used to unequivocally identify the analyte. The need for two procedures was driven by the limitations of available technology. Most determinative methods over the last two decades have been based on liquid chromatography, usually with ultraviolet (UV)/visible or fluorescence detection. Limitations of cost,

availability, and technology prevented the regulatory use of mass spectrometry for quantitation. Even with recent advances in liquid chromatography/mass spectrometry (LC/MS), the need for separate determinative and confirmatory procedures has not been totally alleviated. Often two separate LC/MS analyses are required, one optimized for quantitation and the other for confirmation.

2.1 Determinative procedures

By definition, the determinative procedure must be able to quantify the concentration of the marker residue. For compounds with a tolerance, it is critical that the analysis be able to determine accurately if the concentration of the marker residue is above or below the tolerance in the target tissue. The CVM guidelines[1] for determinative procedures call for an average recovery $\geq$80% with a coefficient of variation (CV) of $\leq$10% for marker residue tolerances of 100 $\mu g\,kg^{-1}$ or greater and an average recovery of $\geq$60% with a CV of $\leq$20% for marker residues with a tolerance below 100 ppb.

Most determinative procedures are based on chromatographic techniques. Because of the amphoteric nature of most animal drugs, derivatization is necessary for analysis by gas chromatography. Therefore, liquid chromatography with UV or fluorescence detection is usually the method of choice. Although practical quantitative LC/MS has been available since the mid-1990s, sponsors have only recently been proposing quantitative procedures based on this technology. Two factors have contributed to the slow adaptation of quantitative LC/MS procedures: limited availability of LC/MS instrumentation in government laboratories, and the significant lag time between a sponsor developing an analytical procedure and the submission of the procedure to the Agency as part of a proposed regulatory method. In the future, LC/MS will likely become the dominant technique used for the quantitation of drug residues owing to its inherent advantages, including simplified sample preparation procedures.

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2.2 Confirmatory procedures

The ability to identify a drug residue positively and unambiguously is needed to support any legal action that the FDA may take against the person responsible for an illegal residue. Historically, mass spectrometry (MS) has been the method of choice for confirmatory methods. MS provides structural information about the residue and is well suited for working with sample concentrations in the parts per million or billion range that are typically observed in residue analysis. Additionally, both the animal drug industry and the FDA have extensive knowledge and expertise in the use of MS for the confirmation of drug residues in animal products.

The confirmatory procedure should be developed for the same tissues for which the determinative procedure was developed, preferably using the same extraction procedure as used for the determinative portion of the method. Storage and stability data are necessary for dried or liquid sample extracts if MS analyses of the confirmatory samples are to be conducted in a laboratory other than the laboratory of sample preparation. Analytes present in sample extracts must be stable long enough for the samples to be shipped to the MS laboratory and analyzed.

The FDA does not prohibit the use of other techniques that could provide unambiguous structural information such as Fourier transform infrared (FTIR) spectrometry. However, the requirement for relatively large amounts of sample for an analysis has limited the use of such techniques.

Historically, the Agency has allowed the use of orthogonal (mutually exclusive) chromatographic techniques for confirmatory procedures. However, confirmation of residues by multiple chromatographic procedures has been rarely utilized. The use of an independent chromatographic technique is an option of last resort. The FDA has not approved the use of an orthogonal chromatographic technique unless the sponsor demonstrates that the use of more specific techniques such as MS is not a viable option for the residue in question. Since the advent of practical LC/MS systems in the 1980s, no orthogonal chromatographic techniques for confirmation have been approved for regulatory use.

3 Development of methods for regulatory use

A drug sponsor or a government laboratory developing a regulatory method should design the method based on its intended use. The successful validation of a method begins with considering the required elements for a regulatory method and incorporating them from the start of method development.

3.1 Practicability of methods

One of the primary requirements for methods is that it be practicable [Section 512(b)(1)(G)]. A method that cannot be used in Federal laboratories has no value in the protection of the food supply. Method developers should avoid the use of rare or custom-made equipment, prohibitively expensive equipment, untested technologies, or reagents that are not commercially available. For a determinative procedure, an analysis should not exceed two working days, and methods should have a minimum sample throughput of at least six samples per analyst-day.

3.2 Analyte selection

The sponsor of a NADA is responsible for generating the data needed to determine the marker residue, tolerance, and target tissue. Typically, this requires a variety of studies using a radioisotope-labeled drug to generate information on total drug residue concentration, metabolites, and residue depletion. The tolerance for the marker residue is based on concentrations of that residue using the proposed regulatory method without correction for recovery. Because of the manner in which the tolerance is set, the tolerance is a method-dependent value. Therefore, alternative methods developed for a NADA drug with an approved tolerance should have a bridging study. The new method should generate results for the marker residue in a known relationship to those determined using the NADA method.

For non-NADA methods developed for unapproved drugs or unapproved uses of a drug, data required to set a tolerance may not be available. Although some data may exist in the literature to aid in choosing an appropriate residue to monitor, basic information is often lacking. Often, the parent molecule is chosen as the monitored analyte. For the majority of compounds, at least some parent drug residue is assumed to be present in the target tissue. Owing to a paucity of toxicological data for many compounds, the limit of detection for the method is set as low as is practical. The FDA will consider all available data on the metabolism and toxicity of the compound in setting these values.

3.3 Specificity

Specificity is the ability of the method to measure accurately the analyte response in the presence of potential analyte interference that might be expected to be in a sample. Specificity is determined by comparing the detector response of a sample extract containing potential interference (e.g., drug metabolites, other animal health drugs, synthetic intermediates of the target drug, degradation products of the target drug, etc.) with the detector response of a solution containing only the analyte. In addition, chromatographic procedures should be tested using sample extracts from control animals from various regions of the country to determine if regional differences affect the matrix components observed in sample extracts. For guidance, CVM recommends that interferences of no greater than 10% of the response of the marker residue at tolerance (for an analyte with an approved tolerance) be present in control matrix chromatograms.

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3.4 Ruggedness

One of the key aspects in developing a method for regulatory analysis is method ruggedness. The more rugged a method, the less susceptible it is to failure or to excessive variations due to differences in equipment, analyst technique, and other differences that are typically present among laboratories. Several factors contribute to poor method ruggedness: insufficient testing by the developer, excessive method complexity, and a failure of the developer to identify and communicate critical points.

Insufficient testing is one of the major causes of method failure. The amount of data needed to publish a new procedure in a peer-reviewed journal and the procedural detail supplied therein are often insufficient to allow a different user to validate a method rapidly. The developer should evaluate if the method will work using chemicals, reagents, solid-phase extraction columns, analytical columns, and equipment from various vendors. Separate lots of specific supplies within a vendor should be evaluated to determine if lot-to-lot variation significantly impacts method performance. Sufficient numbers of samples should be assayed to estimate the lifetime of the analytical column and to determine the effects of long-term use on the equipment.

A complex method with many steps, compared with a simple straightforward procedure, is likely to have many more critical steps that need to be well defined with

proper control limits. Increased method complexity multiplies the work of the developer in identifying and defining critical steps, and also increases the probability that control points for a critical step will not be identified properly. Although method design is primarily driven by the required method detection limit and resources available to the developer, method developers should consider strategies to minimize the complexity of a method in the early design stages.

The method developer should identify critical points in the method. Frequently, the Youden test[2] may be used to determine if temperature, time, flow rate for solid-phase extraction, weight, volume, and other variables in the method are critical. The developer needs to identify if it is acceptable to take a break during a procedure, length of the break, and steps that need to be completed quickly. Because of differences in background and training between analysts, method developers should not assume that other analysts will perform a technique in the same way as in the developer's laboratory. Often analysts will have different interpretations of simple terms such as 'shake', 'slow', 'complete', and 'fast'.

3.5 Stability

The method developer should evaluate the stability of the analyte in the target tissue after short- and long-term storage (days to months) and through multiple freeze and thaw cycles. This assessment should also include an evaluation of analyte stability in stock solutions at the appropriate concentrations and storage conditions. The developer should consider the stability of the analyte during the extraction procedure and during the time a sample may be on an autosampler awaiting chromatographic analysis. Storage stability should be evaluated for fortified and incurred tissue residues at an appropriate temperature (freezer or refrigerator) for a length of time in excess of the time between sample collection and analysis of the last sample. Freeze–thaw analysis should include at least three cycles. Fortified and incurred residue samples should be frozen for at least 24 h and then thawed unassisted at room temperature. The completely thawed samples should be refrozen for 12–24 h; the freeze–thaw cycle should be repeated two more times and the samples analyzed. If the analyte is determined to be unstable, the number of cycles can be shortened or the freezer storage temperature may be lowered to determine the appropriate stability.

3.6 System suitability

System suitability defines the critical performance characteristics that a method must meet to show that it is being used correctly and can generate acceptable data. Retention times, linearity of calibration curves, peak shape, recovery of analyte from fortified samples, and background in control samples are some of the factors addressed by system suitability. Setting system suitability criteria is often a balancing act. Criteria set either too stringently or not stringently enough can lead to method failure. Overly stringent criteria can create a situation where even a well-qualified analyst is unable to meet the suitability criteria. Overly lax criteria can make poor workmanship acceptable, and allow flawed results to be considered valid. For example, failure to

define the minimum resolution between the analyte and interfering peaks can lead to the analyst accepting results from an analysis in which the peaks had partially merged. The developer needs to determine the performance criteria for the method and base the system suitability on these criteria.

4 Method criteria

Prior to submitting a method for trial, the sponsor should develop data demonstrating the performance of the method. At a minimum, the following sample sets for the target tissue should be evaluated for a determinative procedure:

- five control samples
- five control samples fortified with the marker residue at half the tolerance concentration
- five control samples fortified with the marker residue at the tolerance concentration
- five control samples fortified with the marker residue at twice the tolerance concentration
- 10 incurred tissues (two concentrations with five at each) containing residues between half and twice the tolerance concentration generated by treating animals with the drug.

For confirmatory procedures, the fortified sample sets at half and twice the tolerance are not required.

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4.1 Standards

Standards should be analyzed contemporaneously for both determinative and confirmatory procedures. The method developer needs to describe fully the preparation of all the standards and the calibration procedure to be used, such as calibration prior to sample analysis, interspersed standards, or bracketing standards (confirmatory only).

The use of standards prepared in control matrices is typically not allowed for determinative procedures because control tissues are not routinely available to regulatory laboratories. When a matrix effect alters the spectrum or chromatography of an analyte relative to the pure standard, so that confirmatory criteria cannot be met, a control extract containing standard may be substituted for pure standard. Justification, with CVM concurrence, should be provided for confirmatory methods that use fortified control extracts.

4.2 Precision

The precision of an analytical method is a measure of the variability of repetitive measurements. Contributions from numerous sources affect precision, but the major components are within-laboratory (repeatability) and between-laboratory (reproducibility) variations. Precision is expressed as the relative standard deviation (or CV)

because it is relatively constant over the concentration range of interest. For determinative procedures, CVM guidance allows a within-laboratory CV of $\leq$10% when the tolerance is $\geq$100 $\mu g\,kg^{-1}$. When the tolerance is $<$100 $\mu g\,kg^{-1}$, the within-laboratory CV should be $\leq$20%. To be meaningful, precision should be determined using the exact sample and standard preparation procedures that will be followed in the final method.

4.3 Accuracy

Accuracy (systematic error or bias) expresses the closeness of the measured value to the true or actual value. Accuracy is usually expressed as the percentage recovery of added analyte. Acceptable average analyte recovery for determinative procedures is 80–110% for a tolerance of $\geq$100 $\mu g\,kg^{-1}$ and 60–110% is acceptable for a tolerance of $<$100 $\mu g\,kg^{-1}$. Correction factors are not allowed. Methods utilizing internal standards may have lower analyte absolute recovery values. Internal standard suitability needs to be verified by showing that the extraction efficiencies and response factors of the internal standard are similar to those of the analyte over the entire concentration range. The analyst should be aware that in residue analysis the recovery of the fortified marker residue from the control matrix might not be similar to the recovery from an incurred marker residue.

4.4 Other considerations

Adequate sensitivity should be demonstrated and estimates of the limit of detection (LOD) and the limit of quantitation (LOQ) should be provided. The slope of the calibration line may indicate the ability of the method to distinguish the true analyte concentration. The LOD of a method is the lowest analyte concentration that produces a reproducible response detectable above the noise level of the system. The LOQ is the lowest level of analyte that can be accurately and precisely measured. For a regulatory method, quantitation is limited by the lowest calibration standard. The techniques for these estimations should be described.

The FDA requests that the method exhibit sufficient sensitivity to measure accurately the residue of interest after fortification of the control matrix at half the tolerance concentration. Minimally, the detector response at the tolerance should be at least 10 times the average background response.

Linearity verifies that sample solutions are in a concentration range in which the detector response is linearly proportional to analyte concentration. Current FDA guidelines call for establishing linearity. For regulatory methods, this is generally performed by preparing standard solutions at four or five concentrations, from 30 to 200% of the tolerance.

Linearity is often assessed by examining the correlation coefficient (r) [or the coefficient of determination (r^2)] of the least-squares regression line of the detector response versus analyte concentration. A value of $r = 0.995$ ($r^2 = 0.99$) is generally considered evidence of acceptable fit of the data to the regression line. Although the use of r or r^2 is a practical way of evaluating linearity, these parameters, by

themselves, may be misleading and should not be used without a visual examination of the response versus concentration plot. A determination of standardized residuals may be a better estimate of system linearity.

4.5 Confirmatory procedure criteria

For the confirmatory procedure, it is recommended that the sponsor develop spectral data based on at least three structurally specific ions that completely define the marker residue molecule. These ions may or may not include the molecular ion. The use of water loss and isotopic ions is usually unacceptable and CVM concurrence should be sought when water loss ions or isotopic ions are selected for the confirmatory analysis. The proposed fragment ion structures should be consistent with the fragmentation pattern, and justification for specificity of selected ions or scan range should be included. All confirmation criteria should be specified in the standard operating procedure.

Selected ion chromatographic peak(s) should exceed a signal-to-noise ratio (S/N) threshold of 3 : 1. The technique used for estimating S/N should be included. Criteria for retention time (t_R) matching should be specified. The t_R criteria should not exceed 2% for gas chromatography/mass spectrometry (GC/MS) or 5% for LC/MS, relative to the retention time of the standard. The relative abundance for three structurally specific ions should match the relative abundance of the reference standard within 10% (arithmetic difference, not relative difference). For example, at 50% relative abundance, the corresponding window would be 40–60%, not 45–55%. These guidelines apply to selected ion monitoring. Currently, the FDA is in the process of updating the guidance on the use of MS for the confirmation of drug residues to address issues regarding confirmation using MS/MS techniques and the matching of full-scan and partial-scan spectra.[3] To be acceptable, the confirmatory procedure should confirm the presence of the analyte in all fortified and incurred samples (no false negatives) at or above the tolerance, and fail to confirm the presence of the drug in all control samples (no false positives).

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5 Standard operating procedures (SOPs)

5.1 Determinative procedure

The format for analytical methods proposed as the regulatory method should be clear and should contain all necessary information needed successfully to perform the laboratory steps and calculate the results. The following is a recommended format for a determinative procedure:

A. Title. A descriptive title should be provided.
B. Scope. The analytes measured and the applicable matrices should be included. The reason why the method is being submitted for regulatory evaluation should be explained. The advantages of the method over existing methodology should be included.

C. Principles. The physical and chemical principles of the method should be described. The structure of the new animal drug should be provided.
D. Reagents. All the reagents (including grade) used in the procedure and their preparation should be listed in this section. If the method uses reagents that may be limited in supply or availability, the specific source of these reagents and ordering information should be provided. Any critical sources or types of reagents should be identified.
E. Equipment. The equipment required should be included along with the manufacturer and model information. All equipment should be commercially available. If equivalent equipment is available, it should be listed or criteria provided to judge the acceptability of equivalent equipment.
F. Procedure. The procedure section should unambiguously describe the stepwise preparation of samples, standards, and blanks. Instrumental variables should be described. Weight and volume measurements should include the acceptable range. The procedure should also include methods for any calculations. Procedures should include, but are not limited to, the following recommended elements:

1. Standard preparation
   a. Extraction
   b. Cleanup
   c. Dilution
   d. Other
2. Controls and fortification sample preparation
   a. Blank reagents
   b. Control matrix preparation
   c. Fortification procedure for control matrix
3. Sample preparation
   a. Extraction
   b. Cleanup
   c. Dilution
   d. Other
4. Instrument operating variables
   a. Instrumental configuration
   b. Monitored response
   c. Specific operating conditions
5. Procedure for instrumental analysis of samples, controls, and standards.
6. Calculations: all dilution factors and calculation parameters should be clearly explained. An example calibration curve should be provided.
7. System suitability information. Minimum requirements for instrument acceptability and any critical operating parameters should be identified.

G. Quality control information. All critical points, with recommended control procedures and performance criteria, should be identified. If applicable, stopping point(s) should be indicated. Performance specifications for instruments and standard materials should be included. Recommended actions to be taken if performance does not meet the acceptance criteria need to be provided. Sample handling

instructions and information on the stability of the analyte in the biological matrix and final extracted samples should be included. Any additional items that the user will need to duplicate the performance of the method set by the developer should be included.

H. Safety considerations. The Occupational Safety and Health Administration (OSHA), US Department of Labor, standard entitled 'Occupational Exposure to Hazardous Chemicals in Laboratories' (29 CFR 1910.1450) makes it necessary to address safety issues in the SOP. The standard requires laboratories that use hazardous chemicals to maintain employee exposures at or below the permissible exposure limits specified for these chemicals in 29 CFR Part 1910, Subpart Z. Hazards associated with any specific chemicals used in a method must be addressed so that the user has the information needed to follow the Chemical Hygiene Plan for their laboratory. The method developer should limit the use of hazardous chemicals where feasible. The use of toxic and/or carcinogenic reagents should be avoided or eliminated as much as possible. Additionally, the cost of disposal is increasing and could impact the practicality of a method. Material Safety Data Sheets for the analyte(s) and any unusual or hazardous reagents should be provided for the user.

5.2 Confirmatory procedure

The confirmatory procedure should follow the same format as the determinative procedure, but also include the following additional information:

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1. Method principles should include the technique used for mass spectral data acquisition.
2. Structure and full spectrum of the marker residue should be included.
3. Spectral data based on at least three structurally specific ions that completely define the parent molecule (may or may not include the molecular ion), or more if nonspecific ions are included. Use of water loss and isotopic ions is discouraged.
4. Proposed fragment ion structures, consistent with fragmentation pattern.
5. Justification for specificity of selected ions or scan range.
6. Instrument operational settings. This includes settings such as zone pressures, temperatures, voltages, and flow variables. If alternative instruments may be used, their operational variables should be listed.
7. Confirmation criteria specified in advance.
8. Operational criteria for repeat injection of the same sample.
9. Estimate of concentration limits for confirmation in matrix.

5.3 Other considerations

At least one negative control and one positive control should be run each day. The positive control should meet recovery or confirmation criteria. The negative control should have no interferences greater than those specified in the determinative

procedure and must fail confirmation criteria for the day's analyses to be valid. As part of method development, sufficient blanks or negative controls should be analyzed after standards or positive samples to ensure that carryover does not cause a false-positive result. If necessary, the indicated number of blanks to be inserted between samples should be added to the SOP.

The method should define all criteria used to determine if an analysis is valid and the data are acceptable as part of the SOP. The analyst may not substitute or modify criteria used to determine the acceptance of data after an analysis has been completed.

6 The method trial

6.1 Second analyst/laboratory check

Prior to a method trial, the FDA strongly recommends that a second analyst or independent laboratory perform the method. The independent analyst is asked follow the method SOP as written. This analyst should not have been involved in developing the method or be familiar with it in any way. The purpose of the independent analysis is to determine if a qualified chemist can perform the method described without input other than that provided in the written instructions. This 'trial run' will typically identify problems with the SOP that are not apparent to the method developer. Although not required by the FDA, the independent assessment can identify potential problems with the method SOP prior to the lengthy and costly method trial. A 'trial run' offers the method developer an opportunity to correct problems and to increase the probability that subsequent method trials will be successful. Finally, the method developer should realize that the variability achieved in his/her laboratory is often less than that realized by less experienced analysts. If a method cannot achieve a suitable degree of repeatability in the developer's laboratory, it should not be expected to do any better in other laboratories.

6.2 FDA review

The FDA reviews a method prior to trial to ensure that the data submitted by the sponsor support the conclusion that the method is suitable for trial. The sponsor should include the following: (1) a complete stepwise, unambiguous description of the method including reagents, apparatus, sampling procedures, preparation of standards and analytical samples, storage conditions, and identification of critical steps and/or stopping points; (2) system suitability criteria to verify and maintain method performance; (3) a typical calibration curve; (4) individual and summary results derived from control, fortified, and incurred residues in the matrix showing that the method meets the specificity, precision, and recovery requirements; (5) raw data and intermediate results including relevant worksheets, calculations, chromatograms, statistical analyses, mass spectrograms, selected ion monitoring data, etc., from the analyses of control, control fortified, and incurred target tissues.

6.3 Inter-laboratory method trial

The method trial process for NADA methods is different to the process for non-NADA methods. However, the validation protocol followed by the participating laboratories and the requirements for acceptance of the method are the same. The trial process also differs for determinative procedures and confirmatory procedures. Determinative procedures are evaluated using the multiple laboratory process, whereas the confirmatory method needs to be evaluated only in a single government laboratory.

The evaluation of all NADA analytical methods was previously conducted exclusively by the CVM. Since 1995, the CVM has offered sponsors of NADA residue methods the option of conducting the method trial through a Sponsor Monitored Method Trial (SMMT) process. The SMMT is conducted according to CVM specifications with CVM oversight. The resultant performance data must be reviewed and judged acceptable by CVM before the method is approved.

In the SMMT process, draft protocols are reviewed, and guidance provided to the sponsor to help ensure that the format and specifications are adequate. The protocol should be approved by CVM prior to the initiation of the method trial. Once the protocol and method description are acceptable to CVM, the methods are sent to the participating laboratories for review, and a method demonstration is scheduled. The method demonstration, attended by all participating laboratory analysts, involves review of the study protocol and method SOP and a laboratory demonstration of the method. Ideally, all revisions are completed by the end of the demonstration and the study protocol is signed.

At a minimum, the method will be tested in one FDA laboratory and two contract laboratories selected by the sponsor. If the method is for a new animal drug in tissue regulated by the United States Department of Agriculture (USDA) as part of the meat inspection program, a Food Safety and Inspection Service (FSIS)/USDA laboratory will be included if sufficient resources are available. The method trial will be conducted using control and incurred target tissues that are supplied by the sponsor. The sponsor may, on request, supply new or unusual reagents or standards.

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Each of the three laboratories analyzes the same sample sets that the developer was required to analyze during method development:

- five control samples
- five control samples fortified with the marker residue at half the tolerance concentration
- five control samples fortified with the marker residue at the tolerance concentration
- five control samples fortified with the marker residue at twice the tolerance concentration
- 10 incurred tissues (two concentrations with five at each) containing residues between half and twice the tolerance concentration generated by treating animals with the drug.

If a separate confirmatory procedure is necessary, the analysis will be conducted in an FDA laboratory. The sponsor may have one or more of the contract laboratories test

the confirmatory procedure, but the confirmatory procedure must pass in the Federal laboratory for acceptance. Each of the laboratories analyzes the following samples:

- five control samples
- five control samples fortified with the marker residue at the tolerance concentration
- 10 incurred tissues (two concentrations with five at each) containing residues between half and twice the tolerance concentration generated by treating animals with the drug.

During the conduct of the method trial, participating laboratories are instructed to use the method as written. Because the goal of the trial is to evaluate the method as written in the SOP, one of the major challenges is to ensure that the participants do not try to improve or modify the method. Minor modifications to accommodate available equipment are allowed in the contract laboratories with the concurrence of the method sponsor. Any deviations from the method by the contact laboratories are to be reported to the sponsor's Study Director for the trial, the CVM method trial coordinator, and are to be listed in the study final report. Modifications or deviations conducted by the government laboratory(ies) will require the concurrence of the CVM method trial coordinator, the FDA reviewer overseeing the conduct of the trial. The trial is conducted in three phases. At the completion of each phase, the Study Director for the trial, or the CVM trial coordinator, reviews the results and gives the analysts in the participating laboratories the clearance to proceed to the next phase.

In the first phase, the performance of the instrumentation used for the method is demonstrated. Based on the analysis of standards, results from the participating laboratory should meet the system suitability requirements of the method. Successful completion of this phase will qualify the analyst, his or her equipment, and the laboratory for the trial. Failure in the first phase does not usually cause a method to fail the trial. However, it can slow the process. When a procedure fails during the first phase of a trial, the sponsor may need to write a cautionary note in the SOP discussing recommended or inadequate types of instruments. To correct the problem, the participating laboratory analyst can substitute equipment that gives adequate performance; alternatively, the sponsor must find a different laboratory to participate in the trial.

In the second phase, analysts in participating laboratories prepare and analyze a minimum of two control samples and two samples fortified at the proposed tolerance concentration. This phase allows analysts to become familiar with the method before the analysis of samples that will be part of the method validation. Results from the second phase should demonstrate that the control samples are without interference and that the analysts in the participating laboratories can achieve acceptable recovery of analyte from the samples. It is not uncommon for an analyst to have to repeat the second phase several times before adequate results are obtained. Failure at this phase of the trial can cause a method to fail the trial. Often the problems are related to a poorly written SOP that does not adequately describe the procedure.

The third and last phase of the trial is the analysis of the validation samples. All data collected are reported. No results are discarded unless a determinate error can be identified. Any request to repeat the assay of a sample should be approved by

the sponsor's Study Director and/or the CVM method trial coordinator. The analyst completes the analysis of the sample set described earlier in this section.

Following the completion of the trial, each participating contract laboratory provides a report of their results to the method trial Study Director. The government laboratory(ies) provide their results to the CVM method trial coordinator. The sponsor compiles the final results from participating laboratories into a summary report. A final version of the SOP is also provided that includes any revisions made because of observations made during the trial. The summary report, electronic and hard copies of all laboratory results, work sheets, and reports from each of the participating laboratories are sent to CVM for final review and acceptance. This should include electronic copies of all information necessary to verify all of the results.

6.4 Confirmatory procedure method trial

Confirmatory procedures are evaluated differently from determinative procedures because of the different intended uses of the procedure. The primary differences are the testing laboratories and evaluation of the resulting data. Because a confirmatory procedure is needed for legal action, the procedure will be evaluated based on the results obtained in a government laboratory.

Another difference between determinative and confirmatory method trial procedures is the way in which sample extracts are prepared for analysis. Most current methods submitted for review use the same sample extraction technique for both the determinative and confirmatory procedures. In those cases where the same extraction technique is used, the sponsor may provide the prepared extract to the FDA laboratory for analysis. Any problems with the extraction procedure will have been corrected during the determinative method trial.

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The final difference is that the FDA analyst alone makes the recommendation based on the data for the acceptance of the confirmatory procedure. The conclusion of the analyst stating the suitability of the procedure for confirming the presence of the marker residue is sent directly to the CVM method trial coordinator in the Office of New Animal Drug Evaluation (ONADE) and not back to the sponsor as with the determinative procedure.

6.5 Non-NADA method trial

The FDA coordinates the method trial process for non-NADA methods. The sample requirements are the same as for the NADA trials. Non-Federal laboratories such as contract laboratories and State laboratories can participate in the process. For a single-residue method, the minimum numbers of samples and laboratories are the same as for NADA method trials.

Non-NADA methods may be designed to detect multiple residues and they may be designed for use in multiple species. In order to validate these multi-residue methods, modifications to the validation protocol relative to single analyte methods are made. Additional laboratories will participate in the method trial, but the number of samples

analyzed at each facility will be decreased. For example, five laboratories may assay only three fortified samples at each concentration. Incurred residue samples can be blended so that multiple residues will be present in the same sample. Two levels of all incurred residues may not be included. However, each laboratory analyzes more 'blinded' samples than it would in a single-residue method. Because analysts are blinded to sample contents and because samples may contain one or more analytes, data generated from the evaluation of blinded samples for a multi-residue trial clearly demonstrate the suitability of the method for regulatory use.

6.6 Evaluation of data and recommendation for use

Guidelines for acceptability of NADA and non-NADA methods are the same. For the determinative procedure, the criteria described in 'Method Criteria' for accuracy and precision are used to evaluate data generated at participating laboratories. There are no criteria for accuracy in the analysis of the incurred residue samples; however, the overall data set is reviewed to see if there is general agreement between results obtained by contract laboratories and relative to the levels reported in the sponsor's laboratory.

On occasion, results from one of the participating laboratories will fail to meet established acceptability criteria. In those cases, acceptance or rejection of the method is determined by the CVM based on overall method performance. For example, a method that has borderline but acceptable performance for both precision and accuracy at two of three participating laboratories and fails badly at a third laboratory would probably fail. A method that was a borderline failure in one laboratory but easily passed in the other laboratories could be accepted.

For confirmatory methods, the confirmatory procedure criteria described previously should be met. All negative control samples should fail to meet the confirmation standard established in the procedure. All samples fortified at or above the tolerance and all incurred residue samples at or above the tolerance should meet the confirmation standard (to confirm) described in the SOP. It has been argued that it is not necessary for incurred samples containing the marker residue at a concentration below the tolerance to meet established confirmatory criteria. However, failure to confirm the marker residue in these samples may indicate a lack of robustness of the procedure. Any procedure that had this problem would be closely examined to ensure that the method would meet the needs of the Agency.

7 Conclusion

The method trial process is long and involved. The primary purpose of the process is to ensure that the FDA and the FSIS have the tools needed to both monitor the Nation's food supply. An acceptable method allows regulators to take regulatory and/or criminal action against those who illegally use drug products in food animals. Method trials are designed to ensure that the method is sufficiently defined so that it can be successfully used in a government laboratory on short notice by an analyst who may have little or no experience with the procedure. Usually, the analyst in the

sponsor's laboratory will have extensive experience with the method, having tested hundreds of samples as part of the studies done to support the NADA. Even for non-NADA methods, the method developer will have become an expert in the procedure during the development process. A successful method trial tests and enhances a method established in an expert laboratory and establishes a SOP for the method. The goal of the process is to provide government data to support the conclusion that the government will have a practicable method to enforce the Food Drug & Cosmetic Act for the animal drug residues determined and confirmed by the method.

References

1. Guideline for Approval No. 3: General Principles for Evaluating the Safety of Compounds Used in Food-producing Animals. Revised July 1994. US Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Veterinary Medicine, Rockville, MD (1994).
2. W.J. Youden and E.H. Steiner, 'Statistical Manual of the AOAC', AOAC International, Gaithersburg, MD p. 33 (1975).
3. Guidance for Industry No. 118: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues DRAFT GUIDANCE June 6, 2001, US Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Veterinary Medicine, Rockville, MD (2001).

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Validation of analytical methods for post-registration control and monitoring purposes in the European Union

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1 Introduction

In this article, an analytical method is defined as series of procedures from receipt of a sample to final determination of the residue. Validation is the process of verifying that a method is fit for purpose. Typically, validation follows completion of the development of a method. Validated analytical data are essential for monitoring of pesticide residues and control of legal residue limits. Analysts must provide information to demonstrate that a method intended for these purposes is capable of providing adequate specificity, accuracy and precision, at relevant analyte concentrations and in all matrices analyzed.

The principles of validation of residue methods for food, water and soil are generally the same. However, not all procedures and requirements are identical. From the public's point of view, the information on residues in food is probably the most important task. Compared with the other two areas (water and soil), the food sector is characterized by the largest number of regulations and legal limits. Therefore, this overview of validation requirements of enforcement methods will focus on methods for pesticide residues in food.

Methods and analytical results are often classified loosely as quantitative, semi-quantitative or qualitative (screening). These categories do not have well-defined or universally accepted boundaries. Since comparison of residue concentrations with legal limits requires exact quantitative results, the validation of quantitative methods is discussed here.

Method validation guidelines for use in trace analysis have been proposed by various authors, but there is little consistency in the recommended approaches. The general validation guidelines proposed by standards organizations such as ISO (International Organization for Standardization), DIN (Deutsches Institut für Normung; German Institute for Standardization) and others are often not well defined and consequently

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are impractical for the validation of new methods within pesticide registration guidelines. For multi-analyte and/or multi-matrix methods that are typical in pesticide residue analysis, it is likely to be impractical to validate a method based on general standards for all combinations of analyte, concentration and type of sample matrix that may be encountered in subsequent use of the method.

Until 1991, manufacturers seeking authorizations for pesticides had to fulfil country-specific requirements of validation of enforcement methods. The term 'enforcement method' means analytical methods which are developed for post-registration control and monitoring purposes. The harmonization of these requirements was initiated with the European Economic Community (EEC) Council Directive 91/414/EEC and temporarily finalized with the 'Guidance Document on Residue Analytical Methods' SANCO/825/00 rev. 6, dated 20 June 2000 [Santé et Protection des Consommateurs (SANCO)]. The evaluation of validation studies by the competent authority is conducted by comparison of these European Union (EU) requirements with the study results and most often without any practical experience of the method. Some details of this evaluation are discussed below.

In any common market, methods of sampling and analysis can have direct repercussions on its functioning, if samples with residues near a maximum residue limit (MRL) are analyzed with different methods, resulting in somewhat different results and different legal conclusions to the exporting and importing country. To avoid such difficulties, Council Directive 85/591/EEC[1] concerning the introduction of EEC methods of sampling and analysis for the monitoring of foodstuffs intended for human consumption was adopted. In the Annex of this Directive, the criteria which have to be tested for harmonized methods are described. The Technical Committee (TC) 275 of the European Organization for Standardization [Comité Européen de Normalisation (CEN)] which was established in 1991 is dedicated to these EU harmonized methods. Two working groups of TC 275 are dealing with pesticide residue methods. Their validation parameters and criteria are discussed in the second part of this article.

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The official pesticide residue monitoring in the EU is organized individually in the Member States. The numbers of laboratories involved differ significantly between Member States, ranging from one in The Netherlands and Sweden to more than 40 in Germany and Italy. Many of the EU Member States have compiled their own official methods. These compilations are popular in the individual countries, but owing primarily to the different languages they tend to be unavailable to other Member States. For this reason, and based on the variety of national monitoring laboratories, different validation procedures have developed. The third part of this article considers three examples of these procedures.

2 Evaluation of enforcement methods for food provided by manufacturers

2.1 The need for enforcement methods from the applicant

Article 4 of Council Directive 91/414 EEC requests Member States to ensure that a plant protection product is not authorized unless its residues, resulting from authorized uses, which are of toxicological or environmental significance, can be determined

by appropriate methods. Consequently, institutions responsible for the authorization expect the manufacturers to develop enforcement methods. However, in the daily practice of food control laboratories, very often these methods are not applicable, especially if single-residue methods are proposed. The reason for this situation is the inefficiency of the use of single-residue methods if the compliance with hundreds of MRLs must be checked for a single commodity. If pesticides are not covered by multi-residue methods, laboratories testing for this compliance need a limited set of group-specific methods, to analyze readily for these compounds. Unfortunately, most countries and laboratories have developed individual solutions for this problem. Sound guidance for manufacturers regarding the use of standardized elements for other than multi-residue methods cannot be given by the competent authorities.

On the other hand, single-residue methods developed by the applicants give basic information about appropriate cleanup steps and specific determination procedures. In addition, not many laboratories other than those from the applicants are able to test the real solvent extraction efficiency. The reason is that extraction studies need radio-labeled incurred residues instead of fortified samples. Hence enforcement methods provided by the manufacturers accelerate the development of methods which meet the needs of (official) food control laboratories.

Enforcement methods provided by the manufacturer are not generally tested in the laboratories of the European regulatory authorities. Very often, proposed methods are evaluated by assessing the logic of proposed procedures and only for the completeness of validation data. For this 'theoretical' review process, as much information as possible should be available. Recovery data from many validation experiments with different kinds of matrices and the resulting chromatograms of control and fortified samples provide the confidence needed by the referee. In the following sections, the most important aspects of this evaluation will be considered.

2.2 The problem with residue definition

Prior to registration, an agreed commitment to the residue component(s) which should be analyzed does not exist. This is contrary to the situation with residue methods, which are developed after MRL setting. Therefore, to establish an acceptable residue definition is the first step necessary prior to any method development. This residue definition for enforcement methods is based on the results of metabolism studies and may cause serious difficulties, especially if the metabolic pathways of the parent compound are very complex, generating a large number of metabolites.

However, there is no general requirement that enforcement methods need to monitor all metabolites of an active ingredient. The primary purpose of enforcement methods is to detect violations of good agricultural practice. For this purpose, residue levels found in samples from the market (so-called Market Basket Surveys) have to be compared with MRLs, which are derived from residue concentrations found in supervised trials. It is not necessary for this comparison to be based on the total pesticide residue. Most often the choice of a single compound (e.g., parent or primary metabolite) as a marker of the total pesticide residue is more feasible. Method development and the later method application are much easier in that case. Only for intake calculation purposes, e.g., when the daily intake of pesticide residues (calculated from the results

of random monitoring programs) are compared to the acceptable daily intake (ADI), must a correction factor be used. Such a correction is not necessary for the control of Good Agricultural Practice (GAP). Therefore, MRL setting and enforcement method development should focus on the marker compound whenever possible.

An official document containing prerequisites for the use of marker compounds does not exist. However, in the author's experience the following rules may be used as guidance:

- the concentration ratio between that marker compound and the total residue must be known (i.e., which part of toxicologically important compounds is represented)
- the percentage of marker should be independent of factors which are outside the control of the analyst (e.g., a significant influence of pre-harvest interval especially if the marker is not the dominant compound of the total residue)
- the concentration of the marker compound must be sufficient to detect the marker at the appropriate low level, with methodology regarded as 'commonly available'.

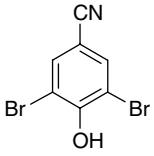
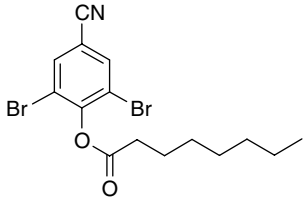
The sensitivity requirement for the use of an individual compound is easily tested. However, the metabolism of a pesticide may change with crop and pre-harvest interval, or may be completely different in animals.

In those cases where the total residue in not represented by a single marker compound, a more complex residue definition is necessary. The hydrolytically unstable ester of bromoxynil octanoate is presented as an example here (Table 1).

Here two components, the free phenol and the intact ester, are included in the residue definition. Usually, analytical methods for the determination of bromoxynil and its octanoate begin with hydrolysis during maceration of the sample. If those methods are validated, the sole fortification of the octanoate is sufficient. However, in other existing methods, hydrolysis follows a separate extraction step. In that case, the chosen solvent must be able to extract both compounds with equal efficiency. As a rule, this has to be tested separately in validation trials. Quicker fortification experiments using a 1 : 1 mixture of both compounds are normally not acceptable. In such experiments an unacceptable extraction efficiency of 40% of one component (e.g., the free phenol) can be masked by a good recovery of 100% of the second component (e.g., the octanoate), resulting in an acceptable total recovery of 70%.

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Table 1 Example of a residue definition based on two compounds

	Bromoxynil	Bromoxynil octanoate
Formula		
Solubility in water (pH 7)	High	Low
Transformation to the second analyte occurs?	No	Yes (by hydrolysis to yield bromoxynil)

To illustrate the potential complexity and difficulties that may occur, the definition of the residue of the fungicide spiroxamine is used as example. In metabolism studies, the amounts of remaining parent compound and individual metabolites were determined after application of spiroxamine, providing the necessary basis for the assessment of dietary exposure, the risk assessment and a proposal of the residue definition. Figure 1 shows the complex metabolism of this fungicide. In the whole plant, straw and grain of wheat spiroxamine and its three main metabolites (see bold labels in Figure 1) contribute 36–91% of the total radioactive residue (TRR). In grain less than 15% of the TRR was identified as unchanged spiroxamine. Greater amounts of the parent compound were detected in the forage only.[2] Unfortunately, a feasible specific method for the determination of spiroxamine and its primary metabolites was not available. However, all these compounds may be hydrolyzed to the common 4-*tert*-butylcyclohexanone moiety. For this reason, the applicant proposed an enforcement analytical method, which determines the parent compound and all metabolites which contain the 4-*tert*-butylcyclohexanone moiety.

In subsequent studies, it was shown that, after hydrolysis, about 42% of the total radioactive residue in grain may be identified as 4-*tert*-butylcyclohexanone. Moreover, 34–90% of the TRR was extracted by heating the plant material under reflux with a mixture of methanol and hydrochloric acid.[3] The performance of that method was properly demonstrated by individual fortification experiments with the parent compound and the three primary metabolites (bold in Figure 1).

However, this residue definition was not approved by the regulatory authority, without considering the remaining large amount of the total residue, which could be determined as a common moiety. The authority recognized that the method could be less feasible compared with the determination of spiroxamine (parent) only. Significant residues in grain are conceivable only if the fungicide is used with a shorter pre-harvest interval, i.e. not under GAP conditions. However, in that case the parent compound becomes the main component of the residue. Therefore, the finally agreed residue definition of spiroxamine was 'parent compound' only.

In summary, the proposal of an appropriate definition of the residue is not a process which follows simple and unambiguous rules in each case. The differences between residue definitions of some European MRLs and US tolerances illustrate the importance of harmonization. However, the great effort sometimes necessary to reach a suitable and accepted residue definition, which considers the needs of risk assessors (toxicologists) and the feasibility aspects of residue analysts, is clearly a vital prerequisite for any method development and validation.

2.3 Elements and format of method description

A standard format for the presentation of analytical methods is not obligatory. However, each study should contain such information normally present in complete method descriptions. The list of key points presented in the SANCO Guidance Document[4] may serve as a guide:

- introduction, including definition of the analyte(s) and scope of the method
- outline/summary of method, including validated matrices, limit of quantitation (LOQ) and range of recoveries and fortifications

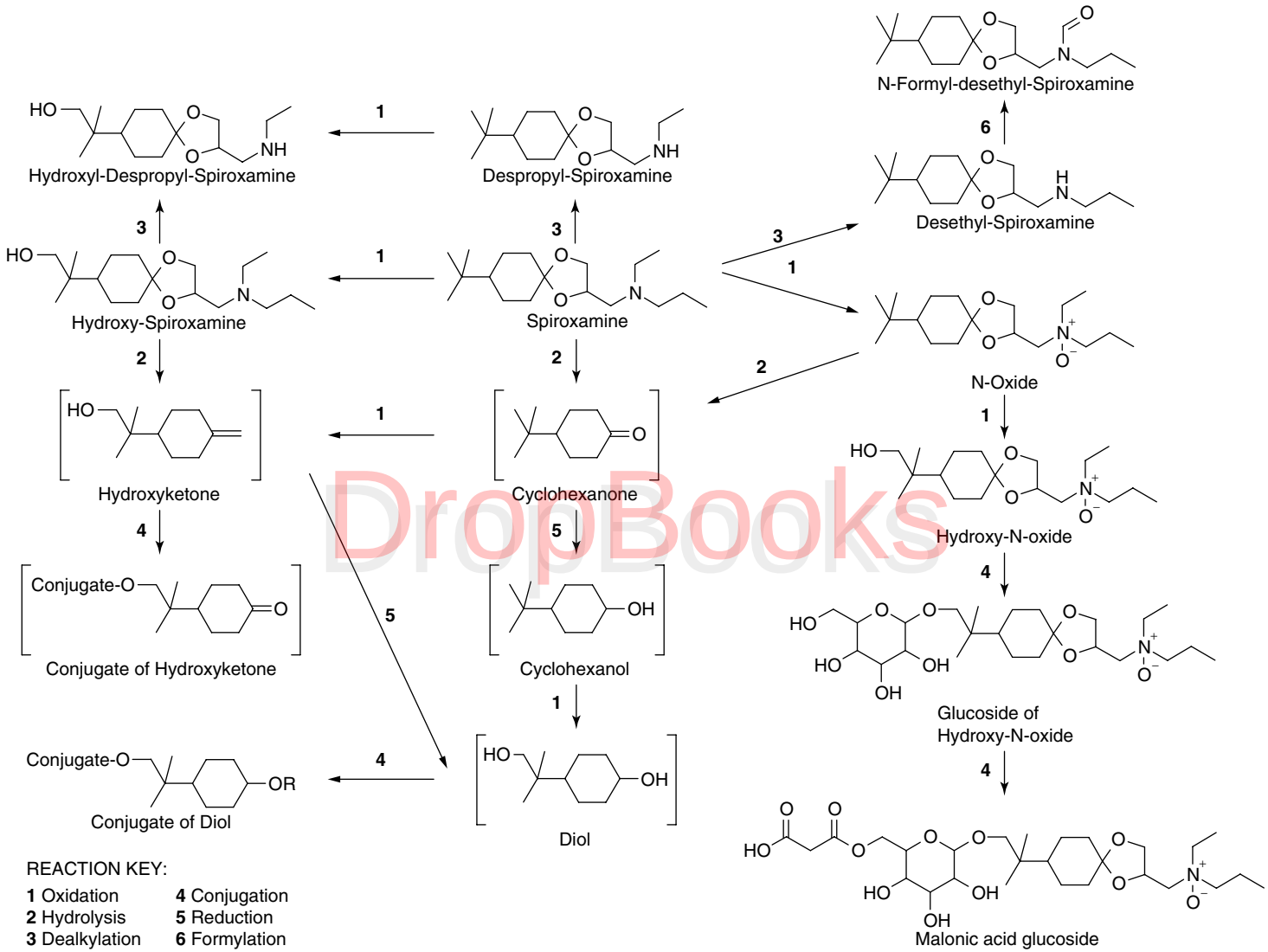


Figure 1 Metabolism of spiroxamine in wheat[2]

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- apparatus
- reagents (including manufacturer and purity as well as full details of the purity of standard compounds and the associated method of determination or clear reference of origin, if commercially available)
- sample preparation (and fortification)
- procedure (extraction, cleanup, derivatization, determination)
- calculation (including typical calibration curves, linearity, correlation coefficient r)
- evaluation (specificity, recoveries, LOQ, repeatability)
- important points and special remarks in analysis (e.g., matrix-dependent deviation, reagent stability)
- representative clearly labeled chromatograms [matrix blank, standard, lowest fortification level (at LOQ) and samples (where possible)] and/or spectra. Labeling should include sample description, chromatographic scale and identification of all relevant components in the chromatogram
- hazards or precautions required
- references.

The following information may be helpful to other method users or referees:

- a statement about extraction efficiency of solvents used
- stability of samples, extracts and standards solutions
- time required for one sample set
- schematic diagram of the analytical procedure.

Most often studies will be accepted by regulatory authorities even if they do not contain all information. For example, a summary, the scope, a separate notice regarding the residue definition or a schematic diagram of the analytical procedure are helpful and may avoid additional questions, but they are not essential. Also, detailed specification of standard glassware or chemicals commonly used in residue analysis is less important. Finally, data about extraction efficiency or analyte stability can be offered in separate studies or statements, which are also valid for other methods. However, each method must precisely describe at the minimum:

- all details to identify the quality and supplier of chromatographic stationary phases and those chemicals which are not commonly used in pesticide residue methods
- the preparation of reagents
- the type and supplier of instruments not commonly used, such as extractors, special cleanup devices, etc.
- the model of gas chromatography (GC) or high-performance liquid chromatography (HPLC) instruments used including the type of columns, detectors and GC injection devices
- all necessary details of alterations/improvement of instruments which are not commercially available (e.g., about the laboratory-made combination of two HPLC instruments with a column switching device)
- all experimental details of extraction, cleanup, identification and confirmation of analyte(s)
- the detailed method of calculation of final results including the calibration procedure(s).

Naturally, the above lists cannot be applied to all circumstances. Sometimes it may be necessary for additional information to be presented.

2.4 Assessment of validation results

To demonstrate the validity of an analytical method, data regarding working range/calibration, recovery, repeatability, specificity and LOQ have to be provided for each relevant sample matrix. Most often these data have to be collected from several studies, e.g., from several validation reports of the developer of the method, the independent laboratory validation or the confirmatory method trials. If the intended use of a pesticide is not restricted to one matrix type and if residues are transferred via feedstuffs to animals and finally to foodstuffs of animal origin, up to 30 sets of the quality parameters described above are necessary for each analyte of the residue definition. Table 2 can be used as a checklist to monitor the completeness of required data.

To avoid any subjectivity in the judgement of performance characteristics presented by applicants, the permitted ranges of several parameters are fixed (e.g., recovery, repeatability, highest intensity of blank signals compared with the LOQ). However, in other cases professional judgement of the referee is required to assess validation results. Some of these aspects are discussed below.

2.4.1 Working range/linearity

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Suitable calibration of instruments used is a fundamental necessity, and it is rarely performed in an appropriate way. Most often, linear calibration functions are regarded

Table 2 Matrix–study combinations for which method validation experiments are needed

Origin of sample	Matrix type	Main study[a]	Independent lab. validation[a]	Confirmatory method[a]
Plant	High water content	a	a	d
	High fat content	a	a, b	d
	High acid content	a	a, b	d
	Cereals and other dry crops	a	a, b	d
	Difficult to analyze	c	b, c	d
Animal	Milk	e	b	d
	Eggs	e	b	d
	Tissue (meat)	e	b	d
	Fat	e, f	b, f	d, f
	Liver/kidney	g	b, g	d, g

[a] (a) May be omitted if use is restricted to another single matrix type. (b) May be omitted if one method is used for all matrix types and two other matrices are validated within the inter-laboratory validation. (c) If use in hops, tobacco, etc., is requested. (d) Often not required for mass spectrometry (MS) or diode-array detection (DAD) methods. (e) When a feeding study is obligatory or MRLs are established. (f) Necessary for fat-soluble active ingredients only (log $P_{OW} \geq 3$). (g) If MRL is proposed or set.

Table 3 Data for a hypothetical calibration curve

Mass injected (ng)	Response (counts)	Response/mass ratio
0.01	30	3000
0.02	100	5000
0.04	270	6750
0.10	800	8000
0.30	2700	9000
1.00	10 000	10 000

as the best choice of calibration. To confirm linearity, the correlation coefficient or the R^2 value is calculated (the different terms 'correlation coefficient' and 'R^2 value' have to be used precisely). Often, a correlation coefficient >0.999 is considered as sufficient proof of linearity.

In the author's experience, such confirmation is not appropriate when the calibration range is greater than one order of magnitude or calibration points are not chosen carefully. The reason is that lower concentration levels of a calibration graph influence the correlation coefficient to a much smaller extent than higher concentrations. The hypothetical example of calibration results presented in Table 3 demonstrates this very simply. If the amount injected is correlated with the observed peak area in the second column in Table 3, the calibration graph in Figure 2 is obtained.

The resulting linear calibration function

$$\text{response} = 10\,105 \times c - 159$$

has a correlation coefficient of 0.99969, which seems to demonstrate linearity. An alternative test for linearity is the calculation of the response/mass ratio. In that case, each observed response (counts) is divided by the amount of standard, which is injected at this level (third column in Table 3). Using the same hypothetical example, a significant deviation from linearity is observed (Figure 3). Consequently, this calculation of the response/mass ratio is more sensitive to nonlinearity in the lower concentration range and is a better test.

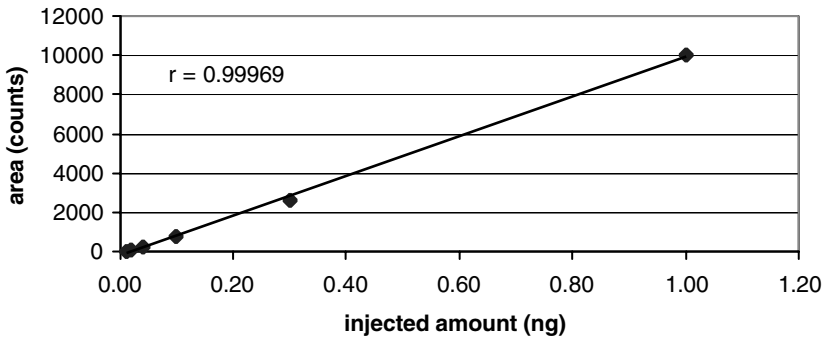


Figure 2 Example of an apparently linear calibration curve drawn from nonlinear calibration data, calculated $R^2 > 0.999$

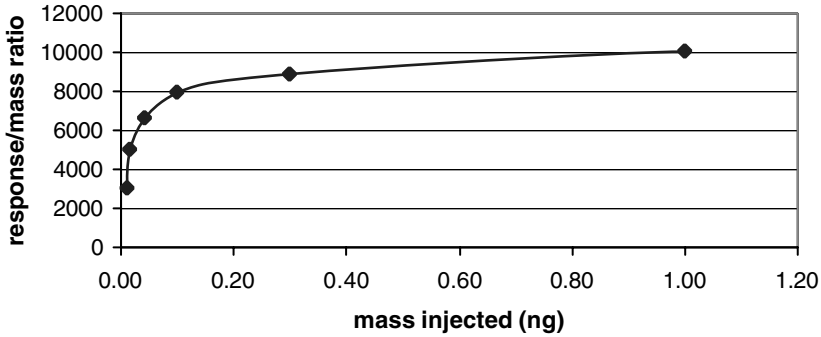


Figure 3 Data for the same hypothetical calibration curve as in Figure 2, calculation of mass response

Finally, a comparison between real and calculated signal intensities can demonstrate the quality of calibration, even if a nonlinear calibration function correlates better with the observed response (Table 4).

Consequently, the proof of calibration should never be limited to the presentation of a calibration graph and confirmed by the calculation of the correlation coefficient. When raw calibration data are not presented in such a situation, most often a validation study cannot be evaluated. Once again it should be noted that nonlinearity is not a problem. It is not necessary to work within the linear range only. Any other calibration function can be accepted if it is a continuous function.

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2.4.2 Specificity and limit of quantitation

The Guidance Document[4] uses a very pragmatic definition for these performance characteristics. The lowest successful validated level is defined as the LOQ. Specificity

Table 4 Comparison of the observed signal intensity with calculated response based on the best fit of a linear or a second-order calibration line

Injected amount (ng)	Observed response (counts)	Calculated response (counts)		Deviation between observed and calculated response (%)	
		Linear calibration[a]	2nd-order calibration[b]	Linear calibration[a]	2nd-order calibration[b]
0.01	30	−58	11	−293	−64
0.02	100	43	100	−57	0
0.04	270	245	279	−9	3
0.10	800	852	821	6	3
0.30	2700	2873	2691	6	0
1.00	10 000	9946	10 001	−1	0

[a] Response = 10 105 × amount−159.
[b] Response = 1213 × amount^2 + 8866 × amount−78.

is based on the absence of interference in the chromatograms of control samples. An LOQ which is calculated by using more sophisticated concepts may be very near to or far away from the lowest validated level. Therefore, the examination of representative chromatograms is essential for both criteria. Often, a definitive confidence in the validity of a method results from the presentation of all relevant chromatograms, i.e., for all matrix types at least chromatograms of one control sample, a sample fortified at the lowest level and a standard solution with an amount of analyte which is equivalent to the lowest fortification level. A typical example which fulfils these requirements is given in Figure 4. However, if the control sample contains more than 15% interference compared with the LOQ, alternatively to the presentation of chromatograms on the same scale, it may be necessary to present the chromatograms of the sample spiked at the LOQ (Figure 4d) and the control sample (Figure 4e) on a scale such that the LOQ peak is about 100% of full-scale.

Tables with quantitation results for all control samples should be presented as supplementary data only and statements similar to '<LOQ' can never replace chromatograms to demonstrate the absence of interference.

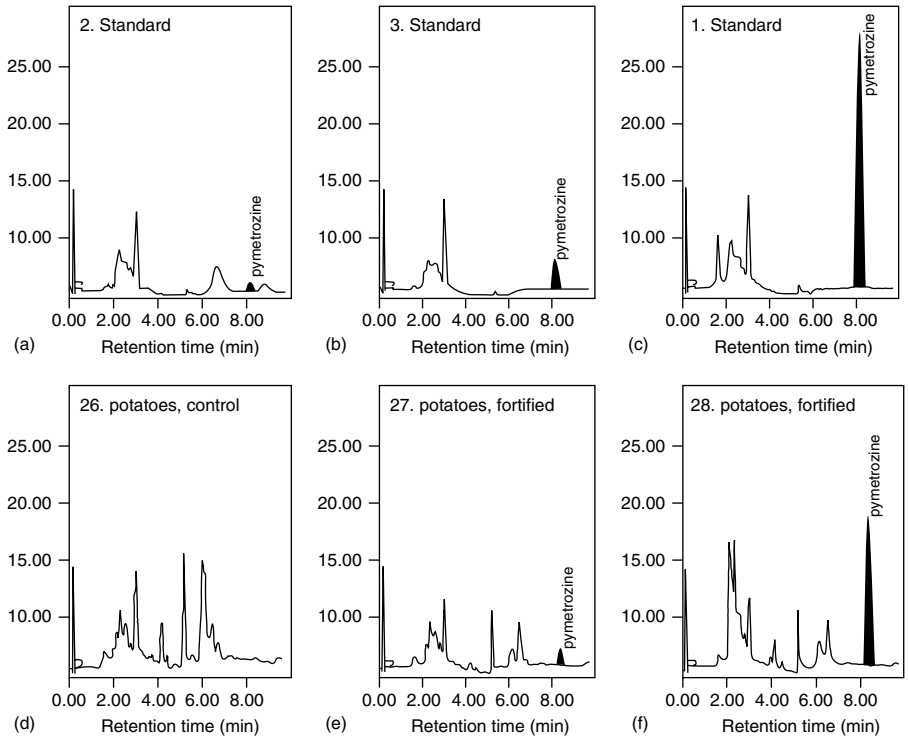


Figure 4 Good presentation of chromatograms obtained during method validation. Top: chromatograms of standards corresponding to (a) 0.01, (b) 0.04 and (c) 0.3 mg kg^{-1}. Bottom: chromatograms of untreated potato samples, (d) unfortified, (e) fortified with 0.02 mg kg^{-1} (= LOQ) and (f) fortified with 0.2 mg kg^{-1}

2.4.3 Other criteria of evaluation

The referee of validation studies has to rely on the performance characteristics provided with the proposed enforcement method. In this context, any deviation from good analytical practice will produce some doubt. The overloading of gas chromatographs, very high sample concentrations (>20 g sample per milliliter of final extract) in extracts without sophisticated cleanup or unusually high injection volumes used in HPLC are problematic and should be avoided. The evaporation of water-containing extracts to a dry residue followed by a partition step between water and an organic solvent does not seem rational. Attributes of good analytical quality are a statement about the response of calibration standards in the presence of a matrix (matrix-matched standards) or the information about those points where a procedure can be interrupted overnight and where it should not. Additional data on the stability or instability of residues in samples, extracts or standards are appreciated. When a special compound is part of residue definition (e.g., a metabolite or common moiety) and not commercially available, a source of that reference compound must be given, otherwise broad application of the method is questionable. Finally, adequate presentation of data can avoid misinterpretations and the need for supplementary statements. Figure 4 and Table 5 are good examples of that presentation.

2.5 Matrices in validation experiments

Although we speak generally of 'validated methods', only the performance of a method applied to a particular range of materials (matrices) is reported. The possibility of matrix interferences or the efficiency of cleanup steps may vary with matrix type. For that reason, methods should be validated in all matrix types, which differ significantly. In the context of the validation of enforcement methods by applicants, 'significant difference' is not a well defined term. To avoid any dispute about completeness of validation, five material types had been selected for crops, which usually

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Table 5 Example for an adequate presentation of blank values, recovery and reproducibility for all matrix types at both levels

Substrate	Fortification level (mg kg^{-1})	Result of control sample (mg kg^{-1})	Recoveries (%, corrected for signal of control samples)	$\bar{x}$	s_{rel}	n
Tomato	0.01	<0.003	116, 112, 115, 115, 112, 107, 105, 104, 103, 109	110	4.4	10
	0.1		95, 101, 100, 102, 98	99	2.8	5
Wheat	0.01	<0.003	101, 103, 113, 95, 107	104	6.5	5
Grain	0.1		100, 100, 98, 96, 98	98	1.7	5
Lemons	0.01	<0.003	101, 99, 109, 105, 101	103	3.9	5
	0.1		99, 105, 102, 98, 96	100	3.5	5
Soybeans	0.01	<0.003	110, 1122, 103, 108, 102	107	4.1	5
	0.1		82, 81, 80, 79, 79	80	1.6	5
Hops	0.1	<0.03	100, 96, 101, 98, 95	98	2.6	5
	1		105, 103, 105, 104, 108	105	1.8	5

should be used in such experiments (i.e., the water-containing, the fatty, the acidic, the dry and, if appropriate, a matrix which is difficult to analyze). Another list exists for materials of animal origin. However, not in all cases must all matrices be used in validation experiments. When a registrant is seeking application in one crop (matrix type) alone, the method performance has to be demonstrated with this matrix only. The necessity for validation of methods for animal materials depends on the occurrence of residues in feedstuffs and animals. The minimum requirements regarding the number of matrix types are listed in Table 2. Additionally, in the author's experience, in some circumstances variations are acceptable:

- Occasionally the complete sample set of an individual commodity was not analyzed within a validation study. This is not a problem if the same study provides data on additional commodities belonging to the same matrix group. Consequently, the missing data, e.g., a second concentration level, are replaced, provided that control sample results are presented for all crops.
- From time to time in older studies, the validity of the method was not tested with all commodity groups. Nevertheless, these studies can be used if the omitted matrix types are tested additionally in the independent laboratory validation.
- The rationale of validation experiments with fatty matrices is the high amount of fat extracted with many organic solvents. If analytes are not fat soluble and extraction is performed with water or aqueous buffer solutions, the troublesome fat is not extracted together with the analyte. Such extractions are typical for, e.g., the class of sulfonylurea herbicides. Examples exist where in such cases the applicability of an analytical method to fatty matrices was accepted by the authority without particular validation.
- Crops with high acid content have to be tested separately, to demonstrate the robustness of methods with regard to changes in pH. In such cases, where extractions are performed at pH values which are lower than those of acidic crops (e.g., <3), the influence of sample acidity is not significant. It is assumed that under such circumstances an expert statement should be sufficient and may replace validation experiments with representative commodities of this matrix group.

The requirements regarding commodities which are difficult to analyze are also not very clear. The listed crops do not cause difficulties in each kind of determination [e.g., brassica or bulb vegetables in gas chromatography/mass spectrometry (GC/MS)]. On the other hand, different species of the same crop may have different interference peaks, which may or may not affect quantitation. Presumably, the easiest approach is to perform additional validations, even if the final extracts are not difficult to analyze. In the author's experience, validations should generally include hops and tobacco, if the pesticide is used in these crops.

A final special case may occur during the validation of common moiety methods. Based on the normal set of recovery experiments (two control samples, five samples fortified at the LOQ and five samples fortified at 10 times the LOQ), in total 12 samples have to be analyzed per matrix and analyte. A typical intention of common moiety methods is their suitability for the parallel determination of residues of the parent compound and a broad spectrum of metabolites. In the common moiety method discussed above for residues of spiroxamine, validation experiments were performed with four compounds. This results in at least 48 experiments per matrix. Assuming a normal

validation, data from four matrix types are required. Consequently, $4 \times 48 = 192$ recovery experiments are necessary. This number of experiments may rise to more than 240 if the intended use includes commodities that are difficult to analyze. Rules to reduce such a workload are not included within the official EU Guidance Document.[4]

On the other hand, some sensible reduction may be acceptable. In the spiroxamine example, an appropriate reduced validation protocol may be as follows: a full set of recovery experiments at both levels performed with the intact spiroxamine (which has the longest reaction pathway to the common moiety) and separately with one primary metabolite. Such two complete validations should be an acceptable test of the working range of the common moiety method.

With the help of fortifications at the LOQ, method sensitivity can be demonstrated for the remaining primary metabolites.

Nevertheless, such special study protocols should be discussed with those regulatory authorities which are involved in the national registration and/or EU evaluation in advance.

2.6 *Test of multi-residue methods*

The Guidance Document on Residue Analytical Methods[4] requests the applicant to assess a standard multi-residue method by using standard steps. These steps are extraction with acetone or ethyl acetate, cleanup by gel permeation chromatography (GPC) and/or silica gel chromatography and final determination by GC.

The best way to test the practicability of the multi-residue approach is to start with the GC determination step. Most often the inability to vaporize the intact compound means that it is not possible to include a new pesticide in the multi-residue scheme. In the case of common moiety methods, a decomposition step is needed to produce the common analyte. Often for that step, modification of the reaction conditions (such as pH and temperature) are necessary, which would lead to a significant deviation from standard multi-residue procedures.

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On the other hand, if only specific GC detectors, e.g. the electron capture, nitrogen–phosphorus or flame photometric detectors, are tested, the argument of lack of GC method sensitivity is not acceptable. In most cases mass spectrometric detectors provide the sensitivity and selectivity needed. Unfortunately, tandem mass spectrometry (MS/MS) or MS^n detectors for GC are still not widely used in official laboratories, and therefore these techniques are not always accepted for enforcement methods.

The elements of the multi-residue method should be used as needed. There is no requirement, for example, to test the full version of the German method DFG 19 without any deviation. This full method combines GPC and silica gel cleanup. A poor recovery of compounds from the silica gel is not a reason to reject the multi-residue approach, provided that the chromatograms of GPC eluates are free from interference.

Occasionally, an additional derivatization step would allow the application of a multi-residue approach. Provided that this derivatization can be done after the standard cleanup, applicants are invited to present those methods. In most cases, for monitoring purposes a supplementary derivatization will be much simpler than a completely separate single-residue method.

2.7 Independent laboratory validation

An independent laboratory validation is the agreed pragmatic procedure to test the reproducibility of a method. First, this practice avoids the conduct of time-consuming and expensive ring tests. Also, minor differences in validation details, e.g., the use of other crops of the same matrix type or small differences in the fortification levels, are permitted. On the other hand, there are disadvantages to this approach. Because of the lowest possible number of independent observations (data from two laboratories) and occasional practical problems (e.g., the inevitable combination of results for different crops), the calculation of between-laboratory standard deviations is meaningless. Consequently, there is no difference in the evaluation of performance characteristics of the independent laboratory validation and the main study. If the working range, recovery, repeatability, specificity and LOQ of the second validation meet the general criteria, acceptable reproducibility of the method is assumed.

The adaptation of an analytical method which is developed in another laboratory often requires some minor modifications. For instance, the use of different models of GC and HPLC instruments cannot be avoided and serves as a test of ruggedness. The use of different GC or HPLC columns is also a normal practice. However, in some cases the independent laboratory is not able to obtain acceptable results without actual modifications of the method. The detection of a significant modification and the differentiation between major or minor modifications are prominent tasks of the examination of the independent laboratory validation data. Significant modifications could include the necessity for additional cleanup steps or the use of different solvents in partition steps. Sometimes, significant differences in the pH of liquid phases follows from minor changes in the amount of reagents used. If no target pH is given, such modification is not acceptable. Such significant modifications require the conduct of a new main study.

By contrast, it is often not possible to standardize cleanup steps based on adsorption chromatography. Altered volumes of elution solvent, small deviations in the water content of the adsorbent and minor changes in the composition of binary eluents are often necessary and should be regarded as minor changes.

2.8 Statement on extraction efficiency

Poor extraction efficiency can be a major source of bias in a method. Rigorous validation of the extraction efficiency of pesticides can only be performed with samples containing residues incurred by the route through which the trace levels would normally be expected to arise in field use. Recovery of analytes from samples fortified shortly before extraction does not necessarily reveal correct information on the extractability of incurred (aged) residues. Suitable certified reference materials containing incurred pesticide residues are rarely available. Occasionally, proficiency test materials with incurred residues are available. However, the storage stability of pesticides in such noncommercial materials is often unknown, complicating the interpretation of results. For this reason, analytical methods validated by routine laboratories may be excellent in the generation of extracts free from interference or may be very selective and sensitive in the final determination step.

Nevertheless, such laboratories cannot remove all doubt about incomplete extraction efficiency.

Validation of true extraction efficiency normally requires the identification and quantitation of 'field-applied' radiolabeled analyte(s), including resulting metabolites and all other degradation products. The manufacturer of a new pesticide has to perform such experiments and is able to determine the extraction efficiency of aged residues. Without any identification of residue components the calculation of the ratio between extracted radioactivity and total radioactivity inside the sample before extraction gives a first impression of the extraction efficiency of solvents. At best, this ratio is nearly 1 (i.e., a traceability of about 100%) and no further information is required. Such an efficient extraction solvent may serve as a 'reference solvent' for any comparison with other extraction procedures.

Often solvents do not extract 100% of the total radioactive residue. In this case, knowledge about the concentration of the target analyte(s) in the extract and the filter cake is necessary. Even if large amounts of radioactivity remain in the solid residual materials, the extraction efficiency may be sufficient if this unextracted radioactivity is permanently bound to the matrix or if it is associated with compounds which are not included in the residue definition. Finally, in all cases a well performed metabolism study can provide the answers needed, even where residues in the edible parts of treated crops or animals do not occur. If incurred residues do not occur, clearly the determination of extraction efficiency is not required.

Consequently, separate experiments for the determination of extraction efficiency are often not required. An expert statement based on the results of metabolism studies is sufficient in most cases. These statements should also refer to the extraction solvent used for the analysis of samples of supervised trials. Residue levels found in these trials are the criterion for GAP and the basis for the setting of MRLs. Even if a solvent with insufficient extraction efficiency is used for samples from supervised trials, the later choice of better solvents would not result in lower safety for the consumer.

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2.9 Perspectives

As discussed before, the efficiency of the extraction step is one of the fundamental performance characteristics of an analytical method. Unfortunately, the provisions regarding extraction efficiency in Council Directive 91/414/EEC (amended by Directive 96/68/EG) are listed in the metabolism section of the directive (Annex IIA 6.1 and 6.2). Nevertheless, results obtained in these studies are essential for the development of enforcement methods and must be reflected in this context.

Better guidance for applicants seems necessary, with the objective that information about extraction efficiency is routinely considered in method validation studies.

Another, often hidden, problem is the matrix effect on the signal response especially of GC and liquid chromatography/mass spectrometry (LC/MS) methods. Despite the requirement that possible effects of the matrix on chromatographic signals must be addressed, such effects are seldom reported. Most often, this matrix effect is investigated only when unexpected high recovery data had been observed by GC. However, the opposite case is relevant for the reliable detection of violations of MRLs, i.e., in fact low recoveries, which pass unnoticed because of the higher intensity of signals of analytes in the matrix compared with the standard in a solvent. The

opposite effect, i.e., signal suppression, is a serious problem especially in LC/MS with electrospray ionization. Fortunately, this matrix effect is combined with an apparently reduced recovery and measures are taken in most cases by the applicant. However, referees of the regulatory authorities as well as applicants should ensure a clear demonstration of the absence of matrix effects or alternatively should prefer the use of matrix-matched standards.

The enforcement methods provided by the applicants give basic information about appropriate cleanup steps and specific determination procedures. Typically, direct use of this developmental work occurred when a GC multi-residue method was found appropriate. Owing to the recent developments in the field of MS/MS with atmospheric pressure ionization, an alternative approach for those compounds that can be analyzed by liquid chromatography (LC) will soon be possible. It is important that some fundamental considerations for such method(s) should be agreed at the outset. Considerations include the most suitable extraction solvents and cleanup steps and some standard HPLC conditions.

Finally, to avoid the parallel use of similar but not identical method validation studies to fulfil the registration requirements, e.g., of the EU, US Environmental Protection Agency (EPA) or Japanese authorities, an adaptation of different data requirements for residue analytical methods for post-registration control and monitoring purposes would help to save resources.

3 Validation of European standard (CEN) methods

3.1 Scope and format of CEN methods

CEN is a legal association, the members of which are the National Standards Bodies of 19 European countries and six Associates, supported by a Central Secretariat based in Brussels. CEN is the European counterpart of ISO. The foremost aim of European standardization is to facilitate the exchange of goods and services through the elimination of technical barriers to trade. The principal aim of CEN is the European standard, which must be published by each of the National Standards Bodies as an identical national standard, with any pre-existing national standards in conflict being withdrawn. The bases for CEN methods are Council Directives 89/397/EEC[5] concerning the official control of foodstuffs and 85/591/EEC of 20 December 1985[6] concerning the introduction of Community methods of sampling and analysis for the monitoring of foodstuffs intended for human consumption. The former Directive refers to the unconditional priority of health and requests harmonization and the effectiveness of official control of foodstuffs. The latter Directive states that methods of sampling and analysis can have direct repercussions on the establishment and functioning of the EU Common Market. They should therefore be harmonized. Methods of analysis which are applicable uniformly to various groups of commodities should be given preference over methods which apply only to individual commodities. (The introduction of the harmonized methods shall not preclude Member States from using other tested and scientifically valid methods. However, in the event of differences in the interpretation of results, those obtained by the use of Community methods shall be determinant.) TC 275 of CEN, which was established in 1991, is dedicated to these horizontal methods. All technical work is progressed within the Working Groups (WGs). Since

Table 6 List of ratified/published standards of CEN/TC 275

Standard No.	Working Group	Title
EN 1528-1:1996	3	Fatty food – Determination of pesticides and polychlorinated biphenyls (PCBs) – Part 1: General considerations
EN 1528-2:1996	3	Part 2: Extraction of fat, pesticides and PCBs and determination of fat content
EN 1528-3:1996	3	Part 3: Cleanup methods
EN 1528-4:1996	3	Part 4: Determination, confirmatory tests, miscellaneous
EN 12393-1:1998	4	Nonfatty food – Multi-residue methods for the gas chromatographic determination of pesticide residues – Part 1: General considerations
EN 12393-2:1998	4	Part 2: Methods for extraction and clean-up
EN 12393-3:1998	4	Part 3: Determination and confirmatory tests
EN 12396-1:1998	4	Nonfatty food – Determination of dithiocarbamate and thiuram disulfide residues – Part 1: Spectrometric method
EN 12396-2:1998	4	Part 2: Gas chromatographic method
EN 12396-3:2000	4	Part 3: UV-spectrometric xanthogenate method
EN 13191-1:2000	4	Nonfatty food – Determination of bromide residues Part 1: Determination of total bromide as inorganic bromide
EN 13191-2:2000	4	Part 2: Determination of bromide

January 1992, two working groups have been dealing with analytical methods for the determination of pesticide residues: WG 3 'Pesticides and PCBs in Fatty Foods' and WG 4 'Pesticides in Nonfatty Foods'. So far analytical methods for 47 pesticides and their metabolites in fatty food and methods for more than 200 pesticides in nonfatty foods have been published in several standards (Table 6).

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The methods EN 1528 : 1996 and EN 12393 : 1998 comprise a range of 'old' multi-residue methods of equal status, which are widely accepted throughout Europe. These are, e.g., the Luke method[7,8] and the German Deutsche Forschungsgemeinschaft (DFG) methods S8[9] and S19[10] (all based on extraction with acetone), the Association of Official Analytical Chemists (AOAC) method 970.52[11] (using acetonitrile extraction and liquid–liquid partition combined with Florisil column cleanup) and the Dutch ethyl acetate extraction combined with GPC.[12] All methods have been subjected to inter-laboratory studies, although not with all pesticide/matrix combinations, which would be impossible to achieve.

Both multi-residue methods are presented in several parts, which separate general considerations from procedures for extraction, cleanup and determination/confirmation. Whereas in EN 12393 several extraction and cleanup steps cannot be combined arbitrarily, the modular concept is utilized to a greater extent in EN 1528. In the latter standard, there is no limitation to the combination of several extraction procedures, mostly designed for different commodities, e.g., milk, butter, cheese, meat or fish, with different cleanup steps. Both standards, EN 1528 and EN 12393, do not specify fixed GC conditions for the determination and confirmation. All types of GC instruments and columns, temperature programs and detectors can be used, if suitable.

The other two CEN standards, for the determination of dithiocarbamate/thiuram disulfide residues and for the quantitation of bromide, are also separated into parts, but, in contrast to the multi-residue methods, complete methods are presented in each different part. Owing to this different approach and the reduced number of analytes, it was possible to validate these methods fully.

3.2 CEN requirements for widely accepted multi-matrix/multi-residue methods

For multi-analyte and/or multi-matrix methods, it is not possible to validate a method for all combinations of analyte, concentration and type of sample matrix that may be encountered in subsequent use of the method. On the other hand, the standards EN 1528 and EN 12393 consist of a range of 'old' multi-residue methods. The working principles of these methods are accepted not only in Europe, but all over the world. Most often these methods are based on extractions with acetone, acetonitrile, ethyl acetate or n-hexane. Subsequent cleanup steps are based on solvent partition steps and size exclusion or adsorption chromatography on Florisil, silica gel or alumina. Each solvent and each cleanup step has been successfully applied to hundreds of pesticides and tested in countless method validation studies. The selectivity and sensitivity of GC combined with electron capture, nitrogen–phosphorus, flame photometric or mass spectrometric detectors for a large number of pesticides are acceptable.

Many experts in Europe have tested the methods of both standards with various pesticide–matrix combinations in their own laboratories. Consequently, the responsible working groups of CEN TC 275 concluded that these are the best methods available. Nevertheless, there is no complete validation of all possible pesticide–matrix combinations. However, for most multi-residue methods within the standards all those pesticides which had been successfully tested in method validation trials and/or proficiency tests are listed. Also, matrices which had been examined in ring tests are listed.

Additionally, under supervision of the Community Bureau of Reference [Bureau Communautaire de Référence (BCR)] the 'Intercomparison Study of two Multi-residue Methods for the Enforcement of EU MRLs for Pesticides in Fruit, Vegetables and Grain' was conducted[13] to support the standardization work within CEN TC 275. About 23 laboratories participated in this inter-laboratory method validation study, which involved a total of 20 pesticides determined in five matrices. In an initial step, these determinations were conducted with fortified samples. Special naturally grown samples with incurred residues were used in the following steps, to compare the extraction efficiency of two solvents. The reports of these projects contain all the usually requested information, i.e., specificity, calibration range, analyte stability during sample storage and processing or analyte homogeneity in addition to the most important parameters, i.e. accuracy and precision. In most cases, the performance characteristics of the methods tested were good, but some method–compound–matrix combinations did not meet all requirements.

In spite of all these efforts, the final responsibility for any analytical results obtained by European Standard methods remains with the analyst who obtained the result. To underline this responsibility, the obligation is included in each standard that 'each laboratory should periodically determine if its results under repeatability conditions are acceptable. . .'.

3.3 Requirements for (newer) methods with limited scope

In contrast to multi-analyte/multi-matrix methods, a more or less complete validation of methods with limited scope is possible. For this reason, TC 275 decided that

methods other than multi-residue/multi-matrix methods must be validated in inter-laboratory method validation studies. The legal basis for this demand is given in Directive 85/591/EEC. In the annex of this Directive, the determination of precision under reproducibility conditions is listed as one of the primary performance criteria. All other parameters of this Directive, that is, specificity, trueness, precision under repeatability conditions, limit of detection (LOD), sensitivity, feasibility and applicability, might be determined in single laboratories, but reproducibility needs an inter-laboratory trial. Another good argument for the need for studies with more than one laboratory is not regulated, but is also important. An inter-laboratory validation is presumably one of the best ways to test the ruggedness of a method, i.e., its ability to resist changes in test results when subjected to minor changes in environmental and method procedural variables.

Within CEN TC 275, a distinct protocol for the conduct of inter-laboratory method validation trials is not selected or required, but it is recommended to apply ISO 5725 or the ISO/International Union for Pure and Applied Chemistry (IUPAC) harmonized protocol.[14] In the past, results of several national and international inter-laboratory trials had been accepted. Nevertheless, the following fundamental aspects of the harmonized protocol for the design of method-performance studies should be fulfilled:

- At least eight laboratories must report results for each material. Only when it is impossible to obtain this number (expensive instrumentation; specialized laboratories required) may the study be conducted with an absolute minimum of five laboratories.
- At least five materials (analyte–matrix–concentration combinations) must be used. Two or more test samples of replicates are statistically considered to be a single material.
- The repeatability precision parameters must be estimated by blind or known replicates.

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In addition, the harmonized protocol provides procedures for the statistical analysis of the data, but ideally, the assessment of data is conducted corresponding to ISO 5725:1994. According to this standard, results are first inspected with the help of two tests developed by Mandel[15] for laboratories which generally did not reach a minimum accuracy or minimum precision. Individual outliers are detected by the Cochran test (repeatability of individual results within a laboratory) and the Grubbs test (difference between the general mean and the mean of an individual laboratory). Note that the confidence levels used for these tests differ between the harmonized protocol and the ISO standard. In each case the Cochran–Grubbs sequence should be reapplied to the data purged of the flagged outliers until no further outliers are flagged or until more than a total of 2/9 would be removed in the next cycle.

3.4 Assessment and documentation of validation results

The assessment of validation data of CEN methods does not differ significantly from other validation schemes. The most important quantitative performance characteristics are trueness and precision. Additionally, some information about sensitivity

and selectivity is available. Trueness is calculated as mean recovery of pesticides from replicate determinations of certified or fortified samples and should fall within the range 70–110%. However, when working near the LOQ, this range may not be achieved.

The precision of recovery is determined under repeatability and reproducibility conditions. The more important 'between-laboratory' reproducibility is calculated as relative standard deviation (RSD_R) and compared with the RSD_R, which is estimated from the Horwitz equation using the same analyte concentration.[16] For good methods this ratio should be about 1, but a method will usually be accepted if the ratio is not larger than 2.

The sensitivity achieved (LOD) is not normally presented. It is recognized that different laboratories determine dissimilar values for this parameter and even within a laboratory the repeatability of the LOD is low. Most often, the lowest validated concentration gives an impression about the lowest levels that can be analyzed generally with acceptable results. A measure of selectivity is the intensity of blank results. This intensity is discussed by the participants of inter-laboratory validation studies. However, results are not reported and limits are not defined by CEN TC 275. The results of method validations of the several multi-residue/multi-matrix methods are not reported in the same way, but newer methods with limited scope generate analogous tables with validation results (as an example, see Table 7).

Table 7 Presentation of the validation results in EN 13191:2000 'Nonfatty foods – Determination of dithiocarbamate and thiuram disulfide residues – Part 1'

	Sample			
Parameter	Witloof chicory	Baby food carrot juice	Baby food apple/banana	Baby food spinach
Year of inter-laboratory test	1994	1995	1995	1995
No. of samples	1	1	1	1
No. of laboratories	12	11	13	11
No. of laboratories retained after eliminating outliers	12	10	13	11
No. of outlying laboratories	0	1	0	0
No. of accepted results	57	50	63	53
Mean value, $\bar{x}$ (mg kg^{-1})	0.26	0.010	0.020	0.033
Repeatability standard deviation, s_r (mg kg^{-1})	0.024	< 0.001	0.002	0.002
Repeatability standard deviation, RSD_r (%)	9.4	8.5	10.8	7.0
Repeatability limit, r (mg kg^{-1})	0.068	0.002	0.006	0.007
Reproducibility standard deviation, s_R (mg kg^{-1})	0.037	0.002	0.007	0.006
Reproducibility standard deviation, RSD_R (%)	14.1	23.2	35.7	17.1
Reproducibility limit, R (mg kg^{-1})	0.102	0.006	0.020	0.016
Horrat value (RSD_R observed/RSD_R predicted)	0.72	0.73	1.23	0.63

4 Validation of official methods of EU member states

4.1 Overview of existing method collections and validation requirements

The 'Pesticide Analytical Manual' of the US Food and Drug Administration or the 'Official Methods of Analysis' of AOAC International are method collections with international recognition. Both compilations are written in English, one prerequisite for its success. Method collections of EU Member States are most often available only in the national languages. For this reason, they are relatively unknown in other Member States. Fortunately, some good collections of official national methods are translated into the English language. The most important are

- the Dutch 'Analytical Methods for Pesticide Residues in Foodstuffs'[17]
- the German 'Manual of Pesticide Residue Analysis'[18]
- the Swedish 'Pesticide Analytical Methods in Sweden'.[19]

In addition, the Internet is now providing official methods or at least information about the method principles. Therefore, a visit to the home pages of the regulatory authorities may be helpful.

Each individual method collection comprises a large number of methods, which often have different validation statuses. For instance, the most important Swedish multi-residue method (based on ethyl acetate extraction, GPC and GC) is validated for many pesticides by four laboratories, but other methods are presented with single-laboratory validation data. Some methods in the Dutch and German manuals were tested in inter-laboratory method validation studies, but others by an independent laboratory or in a single laboratory only.

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It can be concluded that validation procedures for official pesticide residue methods differ between European countries and even within individual countries. Generally applied protocols do not exist. Nevertheless, in the following sections three validation procedures will be discussed. The determination of the recovery of fortified pesticides is a central part of all procedures, although the numbers of analyses required and the criteria applied in the evaluation of results are dissimilar. More important, the concepts of validation are different. The first concept which will be discussed below focuses on single-laboratory validation. In the second no preference is given. The last concept relies only on validation data from more than a single laboratory. These concepts are developed in the UK, the Scandinavian countries and in Germany, although this does not mean that other concepts are not used there. However, these examples may illustrate the diversity of validation procedures which are successfully applied under a variety of circumstances.

4.2 Single-laboratory validation in the UK

In the UK, most official monitoring of pesticide residues is concentrated in the Central Science Laboratory (CSL) of the Ministry of Agriculture, Fisheries and Food. Additionally, this laboratory is responsible for the data which have to be collected in

Table 8 Summary of CSL parameters and criteria for single-laboratory validation of procedures involved in a quantitative method[20]

Procedure	Parameter	Level(s)[a]	No. of analyses required	Criteria for quantitative methods	Comments
Sample processing	Analyte stability	About 5 × LCL	≥5 replicates of each representative commodity, post-processing, and ≥5 pre-processing, if the notional analyte level is not known at this stage	No significant loss of analyte during processing	Processing validated for use with any subsequent procedure. Validation may be specific to analyte and/or sample matrix
	Analyte homogeneity	About 5 × LCL	≥5 replicates of a representative commodity, post-processing	RSD ≤15% (not including analytical variance)	Processing validated for use with any subsequent procedure. Validation applicable to analytes or commodities with similar physical properties
Sample storage	Analyte stability	About 5 × LCL	≥5 replicates at each time point, including time zero	No significant loss of analyte during storage	Storage validated for use with any subsequent procedure. Validation may be specific to analyte and/or sample matrix
Extraction	Extraction efficiency	About 5 × LCL	≥5 replicates of reference material with incurred residues, or ≥5 replicates by the reference procedure and ≥5 of that under test, using any material with incurred analytes	Mean from test procedures within 95% confidence intervals of consensus value of reference material, or ≥the lower 95% confidence level of the reference procedure	May be valid for any subsequent or prior procedure or method. May be specific to analyte and/or commodity
Cleanup and determination	Specificity of analyte detection	At LCL	Ensure measured response is due solely to the analyte. One analysis of each of ≥5 separate blanks of each representative commodity	Identify by MS, or the most specific technique available	Applies only to detection technique. Cut-off concentrations may be identified for different degrees of specificity

Calibration range	LCL to the required maximum level	≥3 replicates at ≥3 levels (≥5 levels for nonlinear systems), at a minimum of two occasions (usually in the presence of matrix)	Sufficiently repeatable response and fit of calibration line to enable accuracy and precision criteria to be achieved	Dilution of concentration acceptable if calibration, accuracy and precision remain so. Data generated during tests of other characteristics may provide this requirement
Accuracy and precision	LCL and accepted limit (e.g., MRL)	≥5 replicates at each level for each analyte/representative commodity combination	Mean recovery 70–110% with RSD ≤10%; results of reference materials within 99% confidence intervals	Where the method does not permit recovery to be estimated, accuracy and precision are those of calibration
Analyte stability in extracts and pure solvents	LCL and accepted limit (e.g., MRL)	≥5 replicates at each appropriate point in time (including zero) and for each representative commodity	No significant change in analyte concentration	Storage times should reflect those likely to be required

[a] LCL: lowest calibrated level for the intended application.

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the UK within the framework of the EU monitoring program (coordinated program of inspections under Article 4 of Directive 90/642/EEC). Therefore, the guidelines for in-house validation of analytical methods for pesticide residues in food and animal feeds which have been published from this laboratory[20] are discussed here. It is assumed that these guidelines are the most appropriate validation requirements for official UK pesticide residue methods at the moment. However, it should be noted that other (older) validation protocols exist.[21,22]

Because of the small number of laboratories involved, validation of UK methods by inter-laboratory study has become impractical in most cases. Even where it is practical, it is usually impossible to validate all pesticide–matrix combinations. Moreover, single-laboratory validation data will have to be generated. Therefore, the CSL guidelines are one of the first that strictly focus on requirements of single-laboratory validation. Some examples of minimum requirements are given in Table 8. Additionally, these guidelines emphasize some other important aspects of validation and contain some new ideas.

4.2.1 Sample processing and storage

Sub-sampling, processing and storage of samples can profoundly influence the homogeneity and potential loss of residues. A test of storage conditions has to be conducted and should include the maximum likely storage period and temperature. To test the homogeneity achieved, initially the pesticide must be heterogeneously distributed in the matrix. Preferably, this is achieved by fortifying some but not all items of a control sample. The exclusion of a potential loss during sample processing may be demonstrated by measuring the recovery of a known mass of analyte deposited on the samples before they are processed. The additional use of a stable co-deposited internal standard reduces this evaluation to a comparison of concentration ratios. Results of storage stability, sample homogeneity and processing stability tests may be applied, with the procedures, to other methods for the same analytes and matrices. Extrapolations of this validation to similar commodities or chemically similar pesticides are possible, but should be used with care.

4.2.2 Extraction efficiency

This validation typically requires samples with radiolabeled analytes. However, alternative approaches are proposed which involve (i) comparison with extraction of samples using a procedure which has been previously validated rigorously, (ii) comparison with extraction of samples by a very different technique or (iii) analysis of a certified reference material. Generally, this validation should be performed with samples containing analyte incurred by the route by which residues would normally be expected to arise. The simplest option (i) requires fully validated and documented enforcement methods provided by the manufacturer of a pesticide.

4.2.3 Lowest calibrated level

This performance characteristic is introduced as a practical alternative to the LOD or LOQ. Not only between laboratories, but even within laboratories, these limits vary

with time, equipment, analyst, etc. Therefore, the determination of the LOD or LOQ is of little value if analytical results are obtained weeks, months or even years after validation. As an alternative, the lowest calibrated level (LCL) is proposed, i.e., the lowest level of calibration standards which were run on an instrument with acceptable response. An individual LCL is determined with each new analytical batch. Therefore, the LCL is a measure of that sensitivity which is demonstrated for a batch. (Note that the highest sensitivity is not needed all the time. In such cases the LCL may be much higher than the actual LOQ. In any case, its determination is more achievable.)

4.2.4 Matrix effect

The effect of co-extracted matrix components on the analyte response in the final determination step should be assessed. Normally, this is done by comparing the response of standards in solvent with 'matrix-matched' standards, i.e., standards prepared in the extract of a control sample without residues. Because matrix effects tend to be inconsistent, the guidelines propose the general use of matrix-matched calibration unless it is demonstrated to be unnecessary.

4.2.5 Performance verification

Ideally, initial validation should incorporate all target analytes and matrices whereas performance verification (the guidelines use the term 'performance validation') is primarily used to support the validity of on-going analyses. To limit the cost and time required before samples are analyzed, the CSL guidelines accept that most initial parameters are calculated from five replicates only (with the exception of calibration with three replicates; see Table 8). Additional data should (and must) be generated during on-going performance verification. In addition, the validation of minor changes of the procedure, the extension to new sample matrices or the extension to new analytes can often be incorporated into performance verification. However, the laboratory manager must take responsibility for ensuring that the validation provided is adequate. Where the performance verification data indicate that method performance is not adequate, the method may be modified as appropriate and subsequently validated.

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In summary, the CSL guidelines can be simply applied in each laboratory and contain very clear instructions. The validated procedures do not focus on the central analytical part only. Important secondary aspects of the whole procedure (sample processing, analyte stability, extraction efficiency) are also considered. For each parameter which is determined, different criteria for the evaluation of quantitative, semi-quantitative and screening methods are given. Here, it should be noted that compared with other guidelines the requirement for the precision of quantitative methods is very stringent (RSD $\leq$ 10%).

4.3 Validation procedures of the Nordic countries

For the Nordic countries, i.e., Denmark, Finland, Iceland, Norway and Sweden, the validation procedures and acceptance criteria for analytical methods are specified in the Procedure No. 4 of the Nordic Committee on Food Analysis [Nordisk Methodik

Komité for Levnedsmidler (NMKL)].[23] The standard presents a universal validation approach for chemical analytical methods in the food sector. This includes methods for the main constituents and also for trace components. Therefore, the NMKL procedure focuses on primary validation parameters, such as specificity, calibration, trueness, precision, LOD or LOQ and does not refer to special requirements of pesticide residue analysis.

Even if most examples and procedures presented apply to in-house validation, the procedure does not distinguish between validations conducted in a single laboratory and those carried out within inter-laboratory method performance studies. A preference for inter-laboratory studies can be concluded from the statement that laboratories should always give priority to methods which have been tested in method performance studies. Within the procedure a profound overview of different categories of analytical methods according to the available documentation and previous external validation is given. For example, if a method is externally validated in a method performance study, it should be tested for trueness and precision only. On the other hand, a full validation is recommended for those methods which are published in the scientific literature without complete presentation of essential performance characteristics (Table 9).

'Verification' implies that the laboratory investigates trueness and precision in particular. Elements which should be included in a full validation of an analytical method are specificity, calibration curve, precision between laboratories and/or precision within laboratories, trueness, measuring range, LOD, LOQ, robustness and sensitivity. The numbers of analyses required by the NMKL standard and the criteria for the adoption of quantitative methods are summarized in Table 10.

It should be noted that all requirements listed in Table 10 apply to an individual analyte–matrix combination. There are no specific demands or proposals for investigations of the influence of various sample materials. This procedure does propose the study of other matrices together with further parameters influencing robustness of the method.

Table 9 Different categories of analytical methods according to the degree of validation and recommended further work[23]

Degree of external validation	Recommended internal validation
The method is externally validated in a method-performance study	Verification of trueness and precision
The method is externally validated but used on a new matrix or using a new instrument	Verification of trueness and precision, possibly also detection limit
Well established, but not tested method	Verification, possibly a more extensive validation
The method is published in the scientific literature and states important performance characteristics	Verification, possibly a more extensive validation
The method is published in the scientific literature without presentation of performance characteristics	The method needs to be fully validated
The method was developed internally	The method needs to be fully validated

Different sample materials often need some adjustment of pesticide residue methods. The insufficient consideration of matrices in the NKML method validation protocol may be a tribute to the wide scope of this standard.

Furthermore, some additional aspects should not be forgotten if this universal standard is applied to validate pesticide residue methods. For example, the requirement that a concentration-dependent signal must be detected in fortified blank samples is not a sufficient test for specificity. The chromatographic response obtained from the standard must be demonstrated to be attributable to the analyte, otherwise signals from a contaminant or a degradation product may be misinterpreted as the pesticide being analyzed for. Another important point when trying out residue methods is the matrix effect on the response. In the description of the calibration procedure of the Nordic Committee guidelines, it is stated that blank samples should be fortified for the preparation of standards. However, a justification is not given and the 'should' may lead to the temptation to make standards in pure solvents. Finally, for the examination of the linearity of the calibration range the NMKL relies on the correlation coefficient. This may be acceptable if the calibrated concentrations are within one order of magnitude, but the MRLs of most pesticides cover a larger concentration range.

In the NMKL procedure, 'verification' is an important aspect of continuous documentation of the quality of on-going analyses performed with validated methods. Regarding this aspect, the standard gives practical guidance to the circumstances under which verification should be repeated. This is, for example, when:

- major instruments are replaced
- new batches of major reagents are brought into use (e.g., new batches of polyclonal antibodies)
- a method is used for the first time by new staff
- a method has been out of use for a long time.

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This type of verification should be distinguished from the periodic performance verification, which monitors performance, e.g., in control charts.

In contrast to many other validation protocols, the description of the NMKL validation process starts with the protocol of planned validation. This protocol should include, e.g., the needs of the client, available equipment, the chemical form in which the analyte occurs (i.e., in pesticide analysis the residue definition), matrix types, the availability of reference materials and the working range. Consequently, an extra paragraph is dedicated to the requirements for the documentation of validation results, which refers to the rules in Section 5.4.4 of EN 45001 (amended by ISO 17025).

In summary, the procedure of the Nordic Committee describes a comprehensive validation protocol, but it is not specially designed for pesticide residue analysis and has no preferences with regard to single- or inter-laboratory validation. Therefore, if it is applied to pesticide residue methods, some specific validation requirements should be added. The procedure clearly lists all necessary steps of validation and adjusts its recommendations to the degree of previous external validation.

Table 10 Summary of NMKL parameters and criteria for validation of procedures involved in a quantitative method[23]

Procedure	Parameter	Level(s)[a]	No. of analyses required	Criteria for quantitative methods	Comments
(Extraction), cleanup and determination	Specificity of analyte detection	At appropriate concentrations	Comparison of at least one blank sample and a sample to which a known amount of pesticide has been added; additional determinations in the presence of pesticides suspected of interfering with the analyte	Absence of interferences	Additionally, a more concentrated extract of the blank may be analyzed in order to demonstrate that no signals occur
	Calibration range	Evenly distributed levels covering the entire working range	(a) Check the applicability of a linear regression using ≥2 replicates at ≥6 levels (b) if nonlinearity is found, the calibration curve should be based on sufficient points to determine the response function accurately	Correlation coefficient $R > 0.999$ for linear calibrations; alternatively the curvature coefficient n of the power curve $R = kc^n$ should be in the range 0.9–1.1 (R = response; c = concentration); no criteria for nonlinear functions	Determinations should be made on blank samples to which the analyte has been added
	Sensitivity	Working range	Calculate the angular coefficient (slope) of the calibration curve	No criteria necessary	Magnitude of the response caused by a certain amount of analyte
	Trueness	At least two appropriate concentrations	5 replicates at ≥2 levels of fortified samples and simultaneously ≥5 replicates of a control sample (not fortified)	No fixed criteria, because requirements on trueness depend on concentration; a mean recovery of 80–110% generally seems sufficient	Alternatively, certified reference materials, samples analyzed with reference methods or proficiency test materials may be applied

Precision	Low, medium and high with regard to the working range	≥ 10 replicates at each level; alternatively replicates within one laboratory or under reproducibility conditions	Calculate the acceptable relative within-laboratory standard deviation using RSD(%) = $1.33c^{-0.1505}$	The calculation of standard deviation may be based on single or duplicate determinations; qualitative methods need other criteria, which are given
LOD	Blank sample	≥ 20 replicates	Three times the standard deviation of the obtained average signal expressed as residue concentration	Alternatively, instrument noise may be used
LOQ	Blank sample	≥ 20 replicates	Ten times the standard deviation of the obtained average signal expressed as residue concentration	Alternatively, instrument noise may be used

[a] LCL: lowest calibrated level for the intended application.

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4.4 Validation of official methods in Germany

In Germany, the federal states are responsible for official food control. In many states more than one approved laboratory could exist. Consequently, a huge number of official laboratories are working in the field of pesticide residue analysis. On the other hand, many laboratories are working with limited personnel. To reduce the necessary workload of method validation for individual laboratories, methods are tested by temporary working groups. In this way, official pesticide residue methods in Germany are tested by more than one laboratory. An overview of German method validation is given in Table 11.

Single-residue methods and other methods with limited scope (e.g., for dithiocarbamates or *N*-methylcarbamates) often are validated in typical method validation trials. The official procedure for such inter-laboratory validation studies was developed in 1983 and is part of the 'Collection of Official Methods under Article 35 of the German Federal Food Act'.[24] This procedure is based on five known replicates analyzed in at least five (preferably ≥ 10) laboratories. In some points the data evaluation procedure differs from international protocols (e.g., ISO 5725[15]), most significantly in tests conducted for the identification of outliers. However, the final calculation of the performance characteristics, trueness, repeatability and reproducibility, is comparable to that in the ISO standard. Before methods are tested in the final ring test, the procedure being applied and verified by the participants. Methods are improved during this previous method tryout, resulting in a better description of important points and greater ruggedness of analytical procedures.

To reduce the effort, another validation procedure is used for extension of the German multi-residue method[25] to a new analyte. Actually, more than 200 pesticides can be analyzed officially with this method, which is the up-to-date version of the better known method DFG S19. A typical validation is performed by at least three laboratories, which conduct fortification experiments at the same three levels with at least four representative matrices. These representative matrices are commodities with high water content (e.g., tomato), fruits with high acid content (e.g., lemon), dry crops (e.g., cereals) and commodities with high fat content (e.g., avocado).

Selectivity and sensitivity of available instruments are tested in all laboratories in the initial step of validation. The crops used for fortification experiments and the concentration levels are identical in all laboratories. Recoveries are determined with all available detection techniques, but after discussion of the results each laboratory selects individually one 'valid' result for each analyte–matrix–level combination. Only this result is used for the calculation of the final mean recovery and standard deviation. Typical criteria for the acceptance of methods are given in Table 11.

Calibration data (e.g., linearity or sensitivity) are not discussed in detail between laboratories, but a typical calibration starts with 50% of the lowest fortification level and requires at least three additional calibration levels. Another point of calibration is the use of appropriate standards. In 1999 a collaborative study tested the effect of matrix residues in final extracts on the GC response of several pesticides.[26] Five sample extracts (prepared for all participants in one laboratory using the German multi-residue procedure) and pure ethyl acetate were fortified with several pesticides. The GC response of all pesticides in all extracts was determined and compared with the response in the pure solvent. In total, 20 laboratories using 47 GC instruments

Table 11 Summary of German parameters and criteria for validation of procedures involved in a quantitative method

Procedure	Parameter	Level(s)[a]	No. of analyses required	Criteria for quantitative methods	Comments
(Extraction), cleanup and determination	Specificity of analyte detection	At LCL	Response obtained from the analyte must be demonstrated as attributable to the analyte in ≥3 laboratories on ≥4 commodities (typical matrices)	Whenever possible tested by MS; relative retention times obtained with other detectors must be comparable (if stationary phases do not differ)	Comparison of relative retention times determined in different laboratories
	Calibration range	Not specified; usually ≥ 4	Not specified but at least one calibration in each sequence	Linearity not required; any valid calibration is accepted	Calibration with standards in matrix strongly recommended
	Sensitivity	At appropriate concentrations	All available detectors should be tested with all fortified samples in ≥3 laboratories	No criteria necessary	Comparison of results in different laboratories
	Trueness	At LCL and two higher levels	≥1 Replicate in ≥3 laboratories at each level; ≥4 commodities (typical matrices)	Mean recovery 70–110%; calculated for each analyte–commodity–concentration combination	Outliers should be removed (the number of deleted suspicious recovery data must not exceed 20%)
	Reproducibility (independent laboratory validation)	At LCL and two higher levels	≥1 replicate in ≥3 laboratories at each level; ≥4 commodities (typical matrices)	RSD_R usually <30%; calculated for each analyte–commodity–concentration combination	Outliers should be removed (the number of deleted suspicious recovery data must not exceed 20%)
	Repeatability and reproducibility (inter-laboratory method validation study)	At appropriate concentrations; not specified	≥5 replicates at each level; participation of ≥10 laboratories desirable but not required (at least ≥5)	RSD_R smaller than twice RSD of Horwitz equation; calculated for each analyte–commodity–concentration combination	Organization and evaluation in accordance with the official protocol[24]
	Robustness	At LCL and two higher levels	Based on the above-specified analyses	≥3 laboratories must confirm the applicability method	Vendors of chemicals, type of analytical columns or instruments, etc., not specified

[a] LCL: lowest calibrated level for the intended application.

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Table 12 Response of 19 pesticides in matrix extracts compared with the response in solvent (response in solvent = 100%; mean of about 40 GC instruments)

Pesticide	Response found in an extract of					
	Apple	Carrot	Tomato	Orange	Wheat flour	Pesticide mean
Acephate	138	138	147	141	136	140
Demethon-*S*-methylsulfone	123	160	145	137	149	143
Dimethoate	122	119	115	121	116	119
Ethoprophos	109	107	105	112	108	108
Fenpropimorph	94	112	102	110	107	105
Fenthion	109	113	116	112	113	113
Iprodione	112	110	120	110	119	114
Malathion	112	113	112	117	112	113
Methamidophos	129	135	138	136	123	132
Methidathion	111	114	118	118	115	115
Mevinphos	128	119	119	130	119	123
Omethoate	118	133	132	127	125	127
Paraoxon	112	113	108	117	113	113
Paraoxon-methyl	134	141	125	125	120	129
Parathion-methyl	111	113	110	110	110	111
Phorate	120	115	111	112	115	114
Phosmet	138	140	128	152	132	138
Phosphamidon1	126	127	123	139	130	129
Phosphamidon2	123	126	124	130	131	127
Terbufos	108	111	113	111	111	111
Matrix mean	119	124	121	125	122	122

participated in this test. With regard to method validation three important results had been recognized: (a) the observed matrix effect often differed widely between pesticides, laboratories and instruments, (b) the mean response of pesticides was generally higher in matrix extracts than in pure solvent (Table 12) and (c) in about 30% of individual determinations the response in matrix exceeded the response in solvent by at least 100% (Table 13).

This ring test had been conducted with specially selected polar pesticides, and therefore the results are not representative of all pesticides. However, irrespective of this, the study clearly showed that validation studies must be conducted with standards in a matrix. In recovery determinations, conducted with standards in a solvent, the analyst cannot be sure that a bad recovery is not masked by matrix effects.

The determination of LOD and/or LOQ is not officially required. In a ring test of more than 10 laboratories initiated in 1992 by the Society of German Chemists [Gesellschaft Deutscher Chemiker (GDCh)] it was shown that these characteristics differ between laboratories by about one order of magnitude, even if the same pesticides are determined in similar matrices with the same method and calculation of the LOD or LOQ is performed in an identical way.

The analyte stability during storage and processing of samples or in standard solutions and extracts is not part of method validation in Germany. Therefore, insufficient stability will not be routinely detected and even then more or less only by chance. Also, separate tests for analyte homogeneity and extraction efficiency were not performed.

Table 13 Percentage of response ratios above 2 (response in matrix extract divided by the response in solvent; data from about 40 GC instruments)

	% Response ratios found in an extract of					
Pesticide	Apple	Carrot	Tomato	Orange	Wheat flour	Pesticide mean
Acephate	44	35	33	35	24	34
Demethon-*S*-methylsulfone	47	44	35	39	39	41
Dimethoate	25	19	18	21	22	21
Ethoprophos	19	13	20	18	16	17
Fenpropimorph	54	34	46	55	24	43
Fenthion	24	23	32	36	19	27
Iprodione	42	21	31	49	47	38
Malathion	17	14	18	18	19	17
Methamidophos	49	31	32	32	34	36
Methidathion	22	19	21	22	25	22
Mevinphos	24	21	20	27	30	24
Omethoate	59	50	31	37	30	41
Paraoxon	21	20	17	31	22	22
Paraoxon-methyl	31	29	20	31	29	28
Parathion-methyl	17	14	15	17	15	16
Phorate	26	22	20	29	21	24
Phosmet	30	31	28	37	32	31
Phosphamidon1	25	20	30	30	24	26
Phosphamidon2	27	23	24	32	27	27
Terbufos	20	18	25	24	23	22
Matrix mean	33	26	27	33	27	29

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In summary, official German analytical methods for pesticide residues are always validated in several laboratories. These inter-laboratory studies avoid the acceptance of methods which cannot readily be reproduced in further laboratories and they do improve the ruggedness of analytical procedures applied. The recently introduced calibration with standards in matrix improves the trueness of the reported recovery data. Other aspects of validation (sample processing, analyte stability, extraction efficiency) are not considered.

4.5 The problem of appropriate documentation of validation data of multi-matrix/multi-residue methods

Any validation and verification work performed must always be documented in such a way that the results can be checked and the scope of a method is clear. International standards, e.g., ISO 17025, contain separate sections regarding documentation, which should be observed. The NMKL procedure on method validation states that 'It is of particular importance that the report includes all raw data from the experimental work, or references to where such data can be found'. In some circumstances this complete documentation is impractical. Even where it is practical, it is usually impossible to publish these results together with the methods.

If analytical methods are validated in inter-laboratory validation studies, documentation should follow the requirements of the harmonized protocol of IUPAC.[14] However, multi-matrix/multi-residue methods are applicable to hundreds of pesticides in dozens of commodities and have to be validated at several concentration levels. Any complete documentation of validation results is impossible in that case. Some performance characteristics, e.g., the specificity of analyte detection, an appropriate calibration range and sufficient detection sensitivity, are prerequisites for the determination of acceptable trueness and precision and their publication is less important. The LOD and LOQ depend on special instrumentation, analysts involved, time, batches of chemicals, etc., and cannot easily be reproduced. Therefore, these characteristics are less important. A practical, frequently applied alternative is the publication only of trueness (most often in terms of recovery) and precision for each analyte at each level. No consensus seems to exist as to whether these analyte–parameter sets should be documented, e.g., separately for each commodity or accumulated for all experiments done with the same analyte. In the latter case, the applicability of methods with regard to commodities can be documented in separate tables without performance characteristics.

As a result of this dilemma, examples of all kinds of documentation exist in official method collections. At the simplest level, it starts with the presentation of tables containing (a) the pesticides and (b) the matrices which can be analyzed, but without any performance characteristics. The most complete documentation is found in the Swedish manual, where the number of experiments, the mean recovery and its range and the RSD are listed separately for each pesticide–matrix–level combination.[27]

In Germany, very detailed documentation is also preferred, but in about 15 pages of validation tables of the German multi-residue method, slightly different levels (concentration differences $\leq$250%) and all matrices with common properties are combined in a single data set.[25] Typical matrices are commodities with high water content, fruits with high acid content, dry crops and commodities with high fat content. This concept of matrix types and the use of representative matrices for validation has recently been accepted by several organizations.[28]

Obviously, a 'best' or generally accepted documentation of performance data of validated multi-residue methods does not exist. Too many data are collected and their detailed presentation may be confusing and impractical. Additionally, the validation of multi-residue methods is a continuous on-going process which started for many pesticides 20 years ago, when less comprehensive method requirements had to be fulfilled. For this reason, a complete and homogeneous documentation of method validation data cannot be achieved.

5 Summary and conclusion

In Europe, very different concepts of method validation are in use. The extent of validation depends upon legal requirements (e.g., for enforcement methods provided by the applicant), upon the required level of acceptance (e.g., for CEN methods) and upon national resources. Undoubtedly, the best method validation is performed with the help of inter-laboratory studies of performance, but such studies can be uneconomic, too slow to reach completion or restricted in scope.

The validation of enforcement methods provided by the applicants for pesticide registration relies on validation trials in at least two independent laboratories. To avoid any additional demands of regulatory authorities after completion of validation, very detailed requirements exist for nearly all circumstances, e.g. if a pesticide is used in a single crop or in more than one crop group (matrix type) or if residues occur in animal tissues or products such as milk or eggs. The solubility of pesticides in fat may also be an important parameter. On the other hand, at that stage MRLs are not set for new pesticides/active ingredients and with the absence of MRLs an official residue definition does not exist. Therefore, the deduction and establishment of an acceptable residue definition is a vital issue for those seeking registration. Owing to the particular interest in feasible methods, data generation methods will not often be accepted as enforcement methods and results of multi-residue methods must be provided.

The integration of analytical methods in European standards requires their acceptance by several national experts within special working groups and in a final weighted vote of National Standards Bodies. Therefore, there needs to be very high confidence in the performance of methods. Consequently, methods should be tested in inter-laboratory method validation studies, with the exception of those multi-residue methods which are widely used throughout Europe. In the case of CEN methods there is no doubt about residue definition but detailed requirements about the number of matrices and concentration levels in validation experiments do not exist. For this reason it may be that CEN methods are validated for important crops only.

The 'workhorses' in national monitoring programs are multi-residue methods. Any official method collection of any EU Member State contains at least one multi-residue method. For multi-analyte and/or multi-matrix methods, it is likely to be impractical to validate a method for all possible combinations of analyte, concentration and type of sample matrix that may be encountered in subsequent use of the method. Therefore, initial validation should incorporate as many of the target analytes and matrices as practicable. For practical reasons this validation and the evaluation of other methods with limited scope often cannot be conducted in inter-laboratory studies. Other concepts based on independent laboratory validation or validation in a single laboratory have been developed and can provide a practical and cost-effective alternative (or intermediate) approach.

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In a widely accepted definition, an analytical method can be defined as the series of procedures from receipt of a sample to the production of the final result. Often, not all procedures can be validated in an adequate way. However, even in such cases, where all procedures of a method are validated, the performance characteristics obtained do not reflect all sources of error. In a recent paper,[29] the complete 'ladder of errors' is described in the following way:

$$\text{result} = \text{true value} + \text{method bias} + \text{laboratory bias} + \text{run bias} + \text{repeatability error}$$

The magnitude of these errors can be analyzed in single laboratories (run bias and repeatability error), in inter-laboratory validation studies (laboratory bias) and in proficiency tests (method bias). Expressed in standard deviations relative to that of

Table 14 Magnitude of analytical errors expressed as standard deviations, relative to that of repeatability

	Level of variation	
	Separate	Cumulative
Repeatability	1.0	1.0
Runs	0.8	1.3
Laboratories	1.0	1.6
Methods	1.3	2.2

repeatability, rough estimates of the four sources of variation are summarized in Table 14.

Table 14 can be regarded as providing a reasonable overall picture, even if the results cannot applied to any particular case. However, if the underlying principle is accepted, it becomes clear that improvements in a single stage, for example the reduction of instrument variation, has a negligible beneficial effect (if this variation was not outside the normal range!). Even if the contribution of repeatability is reduced to zero, the cumulative uncertainty is reduced by 10% only, i.e. from 2.2 to $\sqrt{(0.0)^2 + (0.8)^2 + (1.0)^2 + (1.5)^2} = 2.0$. This statistical view of errors should help to avoid some unnecessary efforts to improve, e.g., calibration. Additionally, this broad view on all sources of error may help to detect the most important ones. Consequently, without participation in proficiency tests, any method validation will remain incomplete.

A final point is the value of earlier (old) validation data for actual measurements. In a study about the source of error in trace analysis, Horwitz *et al.* showed that systematic errors are rare and the majority of errors are random.[30] In other words, the performance of a laboratory will vary with time, because time is related to other instruments, staff, chemicals, etc., and these are the main sources of performance variation. Subsequently, actual performance verification data must be generated to establish method performance for all analytes and matrices for which results will be reported.

References

1. Council Directive 85/591/EEC of 20 December 1985 Concerning the Introduction of Community Methods of Sampling and Analysis for the Monitoring of Foodstuffs Intended for Human Consumption, *Off. J. Eur. Commun.*, **L372**, 50 (1985). Also available on the Word Wide Web: **http://www./europa.eu.int/eur-lex/en/lif/dat/1985/en_385L0591.html.**
2. O. Klein, H. Reiner, and K. Scholz, *Pflanzenschutz-Nachri. Bayer,* **50**(1), 71 (1997).
3. H. Allmendinger, *Pflanzenschutz-Nachri. Bayer*, **50**(1), 49 (1997).
4. Guidance Document on Residue Analytical Methods SANCO/825/00 Rev. 6, Dated 20/06/00, European Commission, Brussels (2000).
5. Council Directive 89/397/EEC of 14 June 1989 on the Official Control of Foodstuffs, *Off. J. Eur. Commun.*, **L186**, 23 (1989). Also available on the Word Wide Web: **http://www./europa.eu.int/eur-lex/en/lif/dat/1989/en_389L0397.html.**

6. Council Directive 85/591/EEC of 20 December 1985 Concerning the Introduction of Community Methods of Sampling and Analysis for the Monitoring of Foodstuffs Intended for Human Consumption, *Off. J. Eur. Commun.*, **L372**, 50 (1985). Also available on the Word Wide Web: **http://www./europa.eu.int/eur-lex/en/lif/dat/1985/en_385L0591.html.**
7. M.A. Luke, J.E. Frohberg, G.M. Doose, and H.T. Masumoto, *J. Assoc. Off. Anal. Chem.*, **64**, 1187 (1981).
8. 'Pesticide Analytical Manual. Volume 1, Multiresidue Methods,' third edition, US Food and Drug Administration Washington, DC, Sect. 302 (1994).
9. G. Becker, 'Method S8, Organohalogen, organophosphorus and triazine compounds,' in "Manual of Pesticide Residue Analysis," ed. H.P. Thier and H. Zeumer, VCH, Weinheim, Vol. 1, pp. 283–295 (1987) and Vol. 2, pp. 313–316 (1992).
10. W. Specht, 'Method S19, Organochlorine, organophosphorus, nitrogen-containing and other pesticides,' in "Manual of Pesticide Residue Analysis," ed. H.P. Thier and H. Zeumer, VCH, Weinheim, Vol. 1, pp. 383–400 (1987) and Vol. 2, pp. 317–322 (1992).
11. 'Organochlorine and organophosphorus pesticide residues, Method 970.52,' in "Official Methods of Analysis of the Association of Official Analytical Chemists," ed. K. Helrich, Association of Official Analytical Chemists, Arlington, VA (1990).
12. 'Analytical Methods for Residues of Pesticides in Foodstuffs,' fifth edition, Rijswiik (1988); amended by 'Analytical Methods for Pesticide Residues in Foodstuffs,' ed. P. van Zoonen, sixth edition, General Inspectorate for Health Protection, The Hague (1996).
13. S.L. Reynolds, R. Fussel, M. Caldow, R. James, S. Nawaz, C. Ebden, D. Pendlington, T. Stijve, and H. Desirens, 'Intercomparison Study of Two Multi-residue Methods for the Enforcement of EU MRLs for Pesticides in Fruit, Vegetables and Grain,' European Commission, BCR Information, Chemical Analysis Contract No. SMT4-CT-95-2030; Reports EUR 17870EN (1997), EUR 18639 EN (1998), EUR 19306 EN (2000) and EUR 19443 EN, European Commission, Brussels (2001).
14. W. Horwitz, *Pure Appl. Chem.*, **67**, 331 (1995).
15. International Standard ISO 5725, 'Accuracy (Trueness and Precision) of Measurement Methods and Results,' first edition, 1994-12-15 Part 2, International Standards Organization, Geneva (1994).
16. K.W. Boyer, W. Horwitz, and R. Albert, *Anal. Chem.*, **57**, 454(1985).
17. General Inspectorate for Health Protection, 'Analytical Methods for Pesticide Residues in Foodstuffs,' ed. P. van Zoonen, sixth edition, Ministry of Public Health, Welfare and Sport, The Hague (1996).
18. Deutsche Forschungsgemeinschaft, 'Manual of Pesticide Residue Analysis,' ed. H. P. Thier and H. Zeumer, VCH, Weinheim, Vol. 1 (1987) and Vol. 2 (1992).
19. 'Pesticide Analytical Methods in Sweden,' Part 1, Rapport 17/98, National Food Administration, Uppsala (1998).
20. A.R.C. Hill and S. L. Reynolds, *Analyst*, **124**, 953 (1999).
21. 'A Protocol for Analytical Quality Assurance in Public Analyst's Laboratories,' Association of Public Analysts, Sheffield (1986).
22. M. Sargent and G. MacKay, 'Guidelines for Archiving Quality in Trace Analysis,' Royal Society of Chemistry, Cambridge (1995).
23. Nordic Committee on Food Analysis, 'NMKL Procedure No. 4, Validation of Chemical Analytical Methods,' NMKL General Secretariat, Finland (1996).
24. 'Planung und Auswertung von Ringversuchen' in "Amtliche Sammlung nach §35 LMBG" (Collection of Official Methods Under Article 35 of the German Federal Food Act), Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin (1983).
25. 'Modulare Multimethode zur Bestimmung von Pflanzenschutzmittel-Rückständen in Lebensmittel "(Modular Multiple Analytical Method for the Determination of Pesticide Residues in Foodstuffs)," in "Amtliche Sammlung nach §35 LMBG" (Collection of Official Methods Under Article 35 of the German Federal Food Act), Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin (1999).
26. L. Alder, G. Kempe, and P. Baumann, 'Need of Matrix Matched Standards–Conclusion from a German Ring Test,' Presented at 3rd European Pesticide Residue Workshop, York, UK, July 3–5, 2000.

DropBooks

27. T. Pihlström, B. Kajrup, and A. Valverde, 'Validation data for 13 pesticides included in the multi-residue method for analysis of pesitcides in fruit and vegetable using ethyl acetate extraction, GPC cleanup and GC determination,' in "Pesticide Analytical Methods in Sweden", Part 1, Rapport 17/98, National Food Administration, Uppsala (1998).
28. AOAC/FAO/IAEA/IUPAC Expert Consultation, 'Guidelines for Single Laboratory Validation of Analytical Methods for Trace-level Concentrations of Organic Chemicals,' Workshop, 8–11 November 1999, Miskolc, Hungary (1999). Also available on the Word Wide Web: **http://www.iaea.org/trc/**(see: pesticides → method validation).
29. M. Thompson, *Analyst*, **125**, 2020 (2000).
30. W. Horwitz, T. Jackson, and S.J. Chirtel, *J. AOAC Int*., **84**, 919 (2001).

Best practices in the generation and analysis of residues in crop, food and feed

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Conducting crop residue field trials in the USA

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1 Introduction

The twentieth century was a period of tremendous change in American agriculture. The development, introduction, and adoption of tractors powered by an internal combustion engine totally changed the way farmers worked, the work they had to do, the time required for them to accomplish the work, and the costs associated with farm production. Along with the introduction of mechanical power and its associated labor-saving tools came new varieties of crops which were resistant to disease, and were locally adapted to environmental conditions so that high productivity was achievable throughout the USA. The introduction of commercial fertilizer and new crop varieties spawned a period of increased productivity unparalleled in agricultural history. This century also saw the introduction of new chemical tools to assist farmers in controlling a myriad of pests (insects, weeds, and fungi) which continued to hamper food and fiber production efforts. Prior to the introduction of these new tools, various types of chemical control agents had been used for many years in the production of some fruits and vegetables. The new, highly effective, synthetic organic chemicals (pesticides) introduced a whole new level of performance and found ready acceptance in nearly all crop production systems. These production practice changes have allowed US farmers to provide the cheapest, most abundant, and highest quality food supply of any nation in the world.

The practical utility of pesticides stemmed from the selective chemical toxicity that existed between the crop and the pest controlled. Since pesticides had the potential to be toxic to other organisms, rules governing their use were quickly introduced. Ultimately the Federal Insecticide, Fungicide, and Rodentacide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA) were enacted into law to regulate this growing agrochemical industry and to monitor the testing required to register a new pesticide. The need for these regulations was based on the awareness that some toxicologically significant residues and metabolites remained on or in the harvested crops that were to be used for food or feed. FIFRA dictated that safe tolerance levels [amount of residue in parts per million (ppm) in/on farm commodities as they leave the farm gate] would be established for these residues, thereby ensuring public safety.

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FFDCA, among other things, assured the safety of processed foods by establishing safe tolerance limits for pesticide residues in processed foods. The rules and interpretation of the rules were not always consistent between these two government offices.

Pesticide registration and use in the USA are regulated by the EPA OPPTS. The regulations are found in the Food and Drug Administration (FDA) Code of Federal Regulations Title (CFR) 40 Parts 152 through 189(1). These guidelines have been revised and updated as new advances in toxicology increased our understanding of the toxic risk pesticides posed. The development of highly selective and extremely potent pesticides has encouraged tremendous strides in the capability of analytical chemistry methods associated with detecting residues in farm commodities. These parallel advances in toxicology and analytical chemistry have strengthened the assurance that pesticides can be used safely and efficiently in our farm production programs. The most recent revisions of the testing guidelines occurred in August 1996 when OPPTS published a unified, consolidated, and correlated new 'how-to' guideline entitled 'Residue Chemistry Test Guidelines'.[2] The intent of the new guideline was to harmonize testing procedures for residue chemistry, which includes generating and analyzing field residue samples. The analytical results indicate the amount of pesticide residue remaining in samples at harvest or after processing and are used in setting pesticide tolerances in food and feed and in evaluating dietary exposure potential. The second recent change was the passage of the FQPA in 1996.[3] The FQPA brought tolerance setting in farm commodities and processed foods under the same tolerance setting guidelines. The FQPA dictated the use of a science-based tolerance setting process for the entire food production system. This was the most significant aspect of this regulation as it pertains to field residue trials. Finally, FQPA dictated that tolerances and overall guidelines be periodically evaluated for relevance as the industry and tools change. Another significant change in recent years is the advent of the Internet. Current regulatory information can readily be accessed from many sources even prior to formal publication. A few of the most useful sites relative to planning and conducting field residue studies are listed below:

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- United States Department of Agriculture (USDA)/National Agricultural Statistical Service (NASS) crop production and usage estimates: **http://usda.mannlib.cornell.edu/reports/nassr/other/pcu-bb/**
- NASS home page: **http://www.usda.gov/nass/**
- EPA OPPTS crop matrix menu: **http://www.epa.gov/oppbead1/matrices/matrixmenu.htm**
- National Center for Food and Agricultural Policy: **http://www.ncfap.org/default.htm**
- EPA Registration Eligibility Decisions (REDs) and Interim Reregistration Eligibility Decisions (IREDs) **http://www.epa.gov/pesticides/reregistration/status.htm**
- EPA OPPTS REDs: **http://www.epa.gov/oppsrrd1/op/**
- EPA Food and Feed Crop Dictionary: **http://www.epa.gov/opphed01/foodfeed/-old/lookatX.htm**

Additionally, commodity groups, CropLife America [(CLA), formerly American Crop Protection Association (ACPA)], the Chemical Manufacturers Association

(CMA), and the USDA are excellent sources of information relative to current regulatory activities which will impact both production agriculture and the setting of tolerances to ensure food safety. The purpose of this article is to summarize the key impacts of the 1996 OPPTS 860 Residue Chemistry Test Guideline series as they impact research associated with field production of RAC samples to be used in establishing safe tolerance limits for pesticides used in commercial agricultural production.

2 Description of the different types of field crop residue studies

Residues of pesticides may be found in many places following the application(s) of a pesticide to a crop. Pesticide residues are commonly found on the surface or inside the tissue of treated crops. Residues may be found in the soil in which the crop was grown. The soil residual materials may arise via either direct application to the soil or from left over plant litter (straw, culls, etc.) which was incorporated into the soil in preparation for the new crop. Residue may be found in following or rotational crops when significant residue remained or accumulated in the soil associated with the treated crop. Residues may also appear in the atmosphere if the product is highly volatile or carried over as spray drift deposits. Finally, residues may appear in run-off water following heavy rain or irrigation or in groundwater if the product and/or its degradation products are highly water-soluble. EPA has established specific testing procedures to address the concentration of the a.i. (parent molecule), metabolic products, and chemical degradation products in the various environmental compartments following the use of a pesticide in the production of a crop. This article will only deal with the residues that are found on or in the plant tissue that will be used for food or feed.

All RACs produced by each crop must be analyzed when establishing a crop tolerance. Specific RAC samples for residue testing have been identified for each crop. The primary commodities include all of the plant parts that may be consumed by people or fed to animals. For example, RAC samples may come from fruits, vegetables, grain, forage, hay, straw, stover, roots, tubers, stollons, bulbs, nut meats, berries, spears, leaves, leaf sprouts, and flower heads. However, the exact samples to be considered in a residue study can be influenced by the label use pattern associated with a specific pesticide and crop. If a pesticide is only applied late in the season, RAC samples that develop prior to the application of the pesticide may not require a tolerance be established. Some crop RACs are commonly converted to processed commodities prior to being eaten (e.g., raisins, grain starch, flour, etc.). Some processing procedures yield by-products that are fed to animals (e.g., raisin waste, wet apple pomace, cotton gin by-products, almond hulls, potato waste, etc.). Residue tolerances, therefore, must be established for each RAC and, where applicable, each processed commodity and/or associated processed by-product.

2.1 EPA guidelines and requirements

The guidelines for field residue trials currently in effect are included in the 'Residue Chemistry Test Guidelines'.[2] The guidelines consist of 17 chapters or sections each

dedicated to specific aspects of the residue chemistry activities associated with obtaining pesticide residue data. For convenience throughout the remainder of this article, these guidelines will be referred to as the 860.Series or as the section number in the series. The actual titles for each of the sections in the 860.Series testing guidelines are as follows:

- OPPTS 860.1000 Background
- OPPTS 860.1100 Chemical Identity
- OPPTS 860.1200 Directions for Use
- OPPTS 860.1300 Nature of Residue – Plants, Livestock
- OPPTS 860.1340 Residue Analytical Method
- OPPTS 860.1360 Multiresidue Method
- OPPTS 860.1380 Storage Stability Data
- OPPTS 860.1400 Water, Fish, Irrigated Crops
- OPPTS 860.1460 Food Handling
- OPPTS 860.1480 Meat/Milk/Poultry/Eggs
- OPPTS 860.1500 Crop Field Trials
- OPPTS 860.1520 Processed Food/Feed
- OPPTS 860.1550 Proposed Tolerances
- OPPTS 860.1560 Reasonable Grounds in Support of the Petition
- OPPTS 860.1650 Submittal of Analytical Reference Standards
- OPPTS 860.1850 Confined Accumulation in Rotational Crops
- OPPTS 860.1900 Field Accumulation in Rotational Crops.

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There are important instructions in each section in the series relative to specific types of tests. However, four sections of the series provide particularly significant instructions relative to field crop residue trials and a short summary of their content is listed below.

2.1.1 OPPTS 860.1000 Background

This section outlines the general intent of the Residue Chemistry Guideline Series and serves as the basic starting point for each of the other sections in the series. In this section the following can be found:

- purpose and scope of data requirements
- regulatory authority upon which the guideline is established
- instructions for minor change in use pattern
- definition of and instructions for food use/nonfood use determinations
- instructions relative to tobacco use tolerances
- considerations for aquatic uses
- special considerations and data requirements for temporary tolerances
- instruction for presentation of residue data
- guidance on submittal of raw data, and references.

Table 1 of this guideline defines the RACs and processed commodities associated with each crop.[1] There is an extensive footnote section to Table 1 that provides considerable additional detail about the crop matrices defined in the table. Table 1 also indicates the percentage of an animal's diet that a particular RAC or processed commodity

must contain if an animal feeding study should be required. The instructions in this section of the guideline should be reviewed early in the planning phase of any crop field residue study.

2.1.2 OPPTS 860.1500 Crop Field Trials

This section outlines the considerations and priorities that were used by the EPA to establish field test guidelines. This section identifies important factors to be addressed in the design, conduct, and reporting of field residue trials. Table 1 indicates the minimum number of trials to conduct and samples to collect in a crop field residue study. The definition and use of crop groups to reduce the field testing cost are outlined in Tables 2–4. At the end of this section is a map that divides the USA into 13 testing or crop production regions, each region representing a fairly uniform farm production environment. This map has been extended into Canada [HED SOP 98.2 Supplementary Guidance on Use of OPPTS Residue Chemistry Test Guideline 860.1500 (residue zone maps – Canadian extension) 4/8/98] and efforts are under way to extend the map into Mexico. The EPA cropping regions in which to locate field residue trials in a study are listed in Table 5. Other important items discussed in OPPTS 860.1500 include:

- the location of the individual trials within EPA cropping regions
- the range of application rates and sample timings that must be included in the study
- how special local needs may be met
- the amount of crop or crop fraction that must be collected to be a representative sample.

Trial number and location and definition of specific crop fractions to be sampled had been a significant reason for study rejection prior to 1996. This particular guideline has helped resolve these issues in studies conducted since that time.

2.1.3 OPPTS 860.1520 Processed Food/Feed

Pesticide residues may be found on the surface of the plant material, or they may be selectively absorbed/translocated inside the tissue. Processing studies are required to determine whether residues degrade or concentrate during typical food processing activities. If residues concentrate during the processing procedures, then a tolerance will be needed for residues in that processing commodity. If residues degrade or do not concentrate, the tolerance for the RAC will be assigned to the food and feed derived from the RAC. Several important instructions relative to the conduct of a processing study as well as preparing and presenting the data from the study are found in this guideline. Additionally, this section provides instructions on how to apply the data to a proposed tolerance when residues are found to concentrate in the processed fractions. Careful attention to the details in this guideline is necessary if a successful processing study is to be conducted.

2.1.4 OPPTS 860.1900 field accumulation in rotational crops

If the confined rotational crop study indicates a potential for residues to persist in the soil and are detected in crops grown as a rotational crop following a treated crop, then a field accumulation study must be conducted.[1] This study is often referred to as a field crop rotation study. The field crop rotation study will provide the data necessary to establish rotational intervals that will limit or prevent residue accumulation in rotational crops. The data may also be necessary to establish residue tolerances for rotational crops that are grown in a normal rotation to the treated crop. This guide becomes particularly important if the confined study indicates residue accumulation at crop rotation intervals of longer than 12 months. This guideline also indicates that intervals of 30 days, 120 days, and 12 months are the standard testing intervals that would be used in setting appropriate restrictions relative to rotation intervals on a particular pesticide use label. If the field crop rotation study indicates there are no residues above the limit of quantitation (LOQ) in rotational crops, then tolerances will not be required for the rotational crop.

Field crop rotation studies are conducted in a tiered fashion. The first tier consists of testing for field residue accumulation in surrogate crops at a limited number (only two required) of sites. A root/tuber crop, a small grain crop, and a leafy vegetable crop (soybeans can be used as a substitute) are used to represent all possible rotational crops. The purpose of this tier is to find a 'plant-back' interval at which a rotational crop could be planted with the expectation that no residue would be found in the RACs. This study can be conducted in a simulated cropping scenario (e.g., treat a primary crop which grows through a normal production cycle before tilling and planting the rotational crop), or the study may be conducted via a simple soil application with the rotational crops planted at desired testing intervals thereafter. The testing strategy to use would be determined by the sponsor's knowledge and anticipation of how the test substance would behave in the normal field environment. If there are no residues in the tier one study at a suitable 'plant-back' interval, no further testing is required. However, if residues are found to accumulate in the tier one study at a desired 'plant-back' interval, then a field accumulation tolerance study is required for each crop that could reasonably be grown in rotation with the treated crop.

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3 Planning phase

The importance of taking the time to develop a viable testing strategy before beginning a field residue project cannot be overemphasized. Failure to plan adequately leads to the most significant complications in actually conducting a field residue study and preparing a final report. Failure to define the project adequately prior to beginning work invariably leads to costly and redundant work and repetition of work in order to reach project goals. During the planning phase of a study, the items described below should all be considered.

3.1 Testing strategy

One of the first decisions that must be made relative to a field residue program is the scope of the overall project. A program for a new development candidate will be

far more complex than a label expansion program. Such questions as the following arise: is the proposed use for a food or nonfood agricultural practice? What crops are to be included and are crop groupings to be used? How many formulations are to be tested? Single a.i. or mixture? How similar are the use rates and patterns between crops? How effective is the candidate in controlling pests within a crop group and over several crop groups? Is the product performance similar over all geographic locations of the USA? Is the intention to obtain a national label or a Special Local Need (SLN) label? How much time is available to complete the work? Can the program be conducted over multiple seasons, or is the program to be conducted within a single season? Once these questions have been adequately addressed, a well-defined testing strategy can then be established which will produce tolerance parameters in the shortest reasonable time and in the most cost-effective manner. If the proposed pesticide use is deemed to have a strong likelihood of not resulting in residues in food, a nonfood use may be considered, and a tolerance will not be required (OPPTS 860.1500). If residues are anticipated in any food or drink (to include eggs, meat, and dairy products), the use is considered a food use. All food uses will require residue trials, and tolerances must be established for the use of the product on each crop.

3.2 Crop and crop grouping

The crop to which a pesticide is applied in a field crop residue study is the test system for the study. If a pesticide is active against pests in multiple, closely related crops, the determination of residue remaining in representative crops may allow a tolerance to be set for all of the crops in the crop group based on the residue in the representative crops (40 CFR 180.40, OPPTS 860.1500). The actual crop or crop group that will be tested in the field residue study defines the test system for the study. 40 CFR 180.40 indicates that if the product is useful on several crops then registering the product for use on crop groups will minimize the number of actual field crop residue trials that must be conducted to obtain maximum access to the marketplace.[1,2] If the study is to determine residues in rotational or following crops, then unrelated crops may be used for the test system for the study. 40 CFR 180.40 defines the two key considerations that must be met for EPA to be willing to consider residue data from a representative group of crops as equivalent for all of the crops in the crop group for the purpose of tolerance setting. First, the use pattern for the crops in the crop group must be essentially the same [same maximum use rate, same number of applications, same time interval between applications, and the same time interval between last application and harvest, 40 CFR 180.40(e)]. Second, the maximum residue level (tolerance) detected in each of the representative crops of the group must not vary by more than fivefold [40 CFR 180.40(g)]. Alternatively, if a single crop in a crop group does not meet these conditions that crop may be excluded from the tolerance, or an individual tolerance may be established for that crop [40 CFR 180.40(h)]. In the USA, the residue trials can all be conducted within a single year. However, unless there are strong drivers for the work to be done in a single season, some testing economies can be realized by conducting the trials over two seasons. The business model being used for the project will determine if this strategy is reasonable and cost effective.

40 CFR 180.41 identifies the actual crop groups and subgroups that could be incorporated into a testing program to minimize testing expenditures while maximizing access to the marketplace. Nineteen groups have been defined as follows:

- root and tuber vegetables
- leaves of root and tuber vegetables (including both human food and animal feed)
- bulb vegetables
- leafy vegetables (except *Brassica* vegetables)
- *Brassica* (Cole) leafy vegetables
- legume vegetables (succulent and dry)
- foliage of legume vegetables
- fruiting vegetables (except cucurbits)
- cucurbit vegetables
- citrus fruits
- pome fruits
- stone fruits
- berries
- tree nuts
- cereal grains
- forage, fodder, and straw of cereal grains
- grass forage, fodder, and hay
- nongrass animal feeds (forage, fodder, straw, and hay)
- herbs and spices.

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In addition to these groups, a twentieth group called oilseed has been proposed. This same list is utilized for tolerance setting in Canada, and the twentieth group has been formally adopted. Crops not listed in this crop group listing must be treated as individual crops for study planning and tolerance setting.

Since the crops listed are fairly large and inclusive for some of the groups, subgroups have been identified to allow more fine-tuning of a marketing plan which would then drive the actual field residue study plan. Relative to the use of a crop group tolerance strategy, the following questions should be resolved during the planning phase: will crop group testing facilitate more rapid access to the marketplace?; and what will the impact of crop group testing have on the risk cup and final market accessed?[3] The information gained from the resolution of these considerations can then be used to prepare the final study protocol.

In addition to the regulatory guidelines surrounding a field residue study, the actual production practices under which the crop will be grown, the way the pesticide will be used on the crop, and any processing needed for the crop to yield appropriate processed commodities must be known. Since very few organizations are large enough to have individual scientists responsible for each of these issues on every team, key study personnel must often review production practices prior to beginning the study plan. Several resources are available to help with this review. Short crop monographs, a summary of crop group implications to residue testing, and a copy of the EPA field residue testing guideline for crop residue studies are found in 'Food and Feed Crops of the United States'.[4] Reviewing more detailed production practices in a standard agronomy[5] or horticulture textbook[6] may be helpful. A very useful reference to help

understand the processing of raw agricultural commodities into food or feed items is 'Foods and Food Production Encyclopedia'.[7] These references provide excellent background information that greatly facilitates planning of a successful field residue study.

3.3 Site/location selection

Table 5 in OPPTS 860.1500 identifies the crop-growing regions in which field residue trials should be conducted.[2] The EPA has identified 13 crop-growing regions in the USA. OPPTS 860.1500 specifies the minimum number and location of tests for each crop in each region. The sites selected for the individual trials in a field residue study should be representative of the agricultural production regions for the crops they represent. For several crops, these locations can be quickly visualized via the maps in 'Agricultural Atlas of the United States'.[8]

Deviation from the 860.1500 test location guideline should be discussed with EPA prior to starting a specialized marketing plan if significant delays are to be avoided during the review process. OPPTS 860.1500 outlines how the number of tests in a study can be modified relative to SLN labels. The use of an SLN may be a particularly useful way to manage unique crop pests found in limited easily definable and reasonably confined production regions.

The number of field trials listed in the various tables of OPPTS 860.1500 are a minimum number of trials to be submitted. More trial locations may be useful or even necessary if specific, unique data will be necessary to defend a proposed tolerance. Including a few extra trials in a field residue study may be advisable to insure that a crop failure during a test season does not diminish the robustness of the study. This practice is particularly important if the entire field residue test program is to be completed in a single growing season. Since some growing regions require a single test, these regions become critical to the success of a study plan. If the study plan allows testing over two seasons, the testing in those regions requiring a single trial should be included in the first season trials. By doing this, potential study failure due to loss of geographical representation when a trial fails will be minimized. Having one or two extra trials in a study to insure against occasional crop failure will assure that the required number of data points are available at the end of the season. This practice would reduce the chance that a study would be inadequate because of crop failure.

Choosing the actual location of a field trial is left to the discretion of the Study Director. The residue data will be most representative of the actual crop production regions if the trials are located within the primary crop production geography for each crop tested. OPPTS 860.1500 indicates the percentage of total US production for each crop grown with the cropping regions identified in the guideline. However, several of the regions are extremely large, and the crops are not grown uniformly over the entire region. Two additional references are useful in defining the final test site selection. The USDA publication 'Agricultural Statistics'[9] identifies the states and counties where the primary production occurs for each of the major crops, and the 'Agricultural Atlas of the United States'[8] plots the production areas by production density dots on a map of the USA. The 'Agricultural Atlas' is published every 5 years

as a result of the census taken in the second and seventh years of each decade. These documents can help confirm that trials in a study have been appropriately located to ensure guideline compliance when the study is completed.

The borders of several of the cropping regions outlined in OPPTS 860.1500 are not the definitive boundaries of the crops produced in that geography. The guideline indicates that when crop production systems straddle one of the boundaries identified in the region map, a test can be placed in either region and count as a trial for either region as long as the cropping system is contiguous in that particular area. However, a trial so defined will only count as one trial for one region and cannot be used to represent both regions in an attempt to reduce the total number of trials conducted. If the registrant wishes to obtain an SLN registration or would like to select a different test location strategy than that listed in OPPTS 860.1500, the use of these additional references to justify the deviation may prove useful.

Another important consideration in field residue trial location is the ability to control environmental events. Access to irrigation can preclude the chance of drought causing crop failure. Location on elevated fields as opposed to flood planes will minimize the chance of damage from flooding. Planting wind brakes (rows of tall crops such as corn, sugarcane, or Sudan grass) can help prevent wind damage to the crop. Physical location and placement of the untreated and treated plots to avoid contamination of the untreated crop during the conduct of the study is also essential. The plots must be located such that wind, rains, or irrigation do not allow movement of the test substance to the untreated plot. Also, the agricultural practices in areas adjacent to the plots must not compromise the integrity of the field trial.

A further important consideration in deciding on the field residue location is making certain that the study protocol is completed in time to allow timely planting of the crop during the normal production system. Some crops are fairly flexible in the conditions under which they grow to produce a desirable crop sample. However, most crops do best when grown under standard temperature, rainfall, and day length cycles. Selection of appropriate locations with good control practices in place can greatly increase the chance of successfully completing the field residue study.

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3.4 Good Agricultural Practice (GAP) and use patterns

The purpose of the field residue study is to produce RACs with residues representative of actual agricultural production practices or anticipated practices associated with the pesticide in question. This necessitates a clear understanding of how the pesticide is to be used during the crop production cycle. In the USA, this has often be called the ‘use pattern’ in the past. With the globalization of agriculture and the harmonization of regulations globally, the term more commonly accepted now is ‘Good Agricultural Practice’ or (GAP). Whichever term is used, the study team should be aware of all of the possible ways the product may eventually be used if the field residue study is to be successful. The method of application, the time of application, time between applications if multiple applications are anticipated (schedule), and the time between the last application and harvest [pre-harvest interval (PHI)] for each RAC associated with a particular crop must be accounted for in the study design. The expectation is that the most severe usage of the pesticide for each RAC will be represented in

the study. The maximum use rate, the shortest interval between applications, and the shortest interval for the PHI must be included in the study design. The study must yield samples representative of the most aggressive possible GAP if the samples are to be acceptable for tolerance-setting purposes. If the product has a simple GAP, then the implementation of the GAP in the study design will be simple. However, if the GAP is complex, the study must be designed very carefully to ensure that all aspects of the GAP are represented relative to all possible RACs of the crop. Failure to do this will result in an unsatisfactory study and the likelihood of the study being rejected or only conditionally accepted until additional trials are completed. Either of these failure scenarios will be costly relative to the field residue testing required. However, the biggest cost to the sponsoring organization will be if a highly desired registration is delayed or denied due to poor representation of the GAP in the field residue trials. Successful design of this portion of the study plan will typically require close collaboration between the Study Director, the registration manager for the product, and the marketing and/or the technical development manager for the product. This trio cannot over-communicate during the design of the study plan. Only if they are working closely together will the GAP be fully understood and clearly represented in the study protocol.

3.5 Test substance

The test substance must be clearly defined in terms of the amount of the a.i. in the pesticide and the formulation type. The test substance used for the field residue program must be identical with the final product for which the registration and marketing license will be requested. If more than one formulation of the a.i. is to be registered, a complete field residue program may be needed for each formulation and each crop (860.1500). However, formulations which are very close in nature may simply need to have bridging studies (limited number of side-by-side field residue studies) completed to demonstrate residue equivalence for the two formulations. If the final GAP will require the use of surfactants or other spray adjuvants in the spray solution, these same spray adjuvants should be included as part of the field residue testing program.

3.6 Residue decline trials

If the RAC of a crop is present at the time of pesticide application, or if quantifiable residues may be present on food or feed commodities near or at harvest, residue decline trials are required (860.1500, p. 16). The primary purpose of these decline trials is to demonstrate whether or not the pesticide residues decline in the RAC over time following the application. For crops requiring 16 or more field trials, two decline trials must be conducted. Crops requiring 5–12 trials require a single decline trial. Crops requiring less than three trials are exempt from decline trials. Decline trials are considered part of the total trial count in meeting the number of trials required for a crop registration. Conducting a few preliminary range finding trials early in a development program may be advisable to understand the nature of the residue decline curve in order to manage the impact of the residue levels throughout the testing process.

Conducting decline trials on all crops that may be treated with a particular pesticide will not typically be necessary. If representative crops demonstrate that residues do not increase with longer PHIs, additional decline trials will not be required for other crops in the representative crop group (860.1500, p. 17). If this approach is used, decline data should be gathered from the five following representative commodities (if they all apply to the pesticide use pattern): a tree fruit, a root crop, a leafy vegetable, a grain, and a fruiting vegetable. The protocol must describe the residue decline strategy for a study if decline data are required.

3.7 Processing study requirement

Some crops are used directly for food or feed while others are processed in some fashion between harvest and actual consumption. Examples of crops and their processed commodities include grapes dried into raisins, plums dried into prunes, apples converted to juice or apple sauce, tomatoes made into juice or catsup (ketchup), wheat ground into flour, soybeans pressed into meal and oil, etc. If the processed commodities of these and other crops constitute a significant food or feed item, then residue tolerances must be set for the processed commodity. The guidance for conducting field residue trials for processed food and feed are found in OPPTS 860.1520. A processing study is necessary to determine whether the residue in an RAC declines or concentrates during the processing procedures. If residues do not concentrate in the various processed commodities, then the tolerance established for the RAC will apply to processed commodities. If the residue does concentrate, then individual tolerances are required for the processed commodities. See the guideline for a detailed description of procedure to follow if this happens. Table 1 in OPPTS 860.1000 indicates which processed commodities are considered significant and, therefore, must be analyzed.

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A single field trial is all that is required to provide the data necessary to establish a tolerance for the processed commodities identified in OPPTS 860.1520. However, one may choose to conduct more than one field trial as insurance against crop failure at a single location which could delay a registration package submittal for another growing season (which would be far more costly to a business than the cost of multiple field trials). Once samples have been collected at one site, other trials could be terminated to minimize overall study cost.

The processing trial should be conducted close to or in conjunction with one of the standard RAC trials. In this way, the residue data from the RAC trial will help confirm the validity of the data obtained in the processing trial. Alternatively, the processing trial could simply be considered as one of the RAC trials, and an additional, larger sample could be harvested for the processing portion of the study. The crop for a processing study should be grown exactly the same as for a normal field residue trial.

Since one of the key purposes of this study is to determine residue partitioning in the various processed commodities, every reasonable effort must be made to start the processing procedures with some level of residue in the RAC. If the RAC has residues present at harvest under normal GAP, then selective partitioning can be easily detected as the RAC is processed. However, if there is no residue in/on the RAC, the guideline indicates that exaggerated application rates may be required to obtain sufficient residue level to conduct a successful processing study. Usually a three- or

five-fold exaggeration in application rate is adequate to meet this requirement. If there is not sufficient residue after a five-fold application rate exaggeration to conduct a processing study, then EPA has indicated that the processing study requirement for the RAC will be waived for that product (860.1520).

Processing studies add one more component of complexity to the study plan. The most successful studies will include representatives from the processing laboratory on the planning phase of the study. The processing laboratory should be informed of the progress of the study, particularly as the study nears completion. The laboratory must be informed of the anticipated timings for the samples to arrive at the processing laboratory. If this is done, the processor will be ready for the processing commodity when it arrives from the field and will be able to generate the processed fractions in a timely manner which most closely represents actual agricultural practices.

The RAC and processed commodities to be collected for each crop are listed in OPPTS 860.1000. Close attention should be paid to the definition and description of many of the commodities listed in the footnotes to Table 1. Reviewing a summary of the actual commercial processing practices for the crop[7] may be helpful. Once the processing procedures and the agronomic practices to be simulated in the field residue trial are understood, a field study can be designed that will truly represent commercial production and processing practices. This will ensure that the study will yield useful, reliable, and accurate data to be used in the tolerance setting process.

3.8 Contract research organizations

With the distribution of tests required for a standard field residue study and the training required for personnel conducting the trials, few organizations currently have the internal staff to conduct these trials independently. The use of highly skilled and specially trained contract research organizations augments internal testing capacity to complete the trials as prescribed by the guideline. Most companies have developed strong relationships with contract organizations or independent principle investigators (PIs) for this collaborative effort. There must be a strong commitment to timely communication between the Study Director and the PI at these organizations. This communication may be via letter, telephone, fax, or e-mail. In recent years, the advent of e-mail has not only facilitated communication between the Study Director and the PI but has provided a convenient way to complete the GLP requirements to confirm these communications. E-mail has quickly become the preferred method of communicating most routine items and is often the most effective way to communicate critical items when the Study Director and PI are located in different time zones. Contract field research organizations operate under two general business models. Some organizations own their own research farms and can operate under very stringent control and, if necessary, secrecy relative to a particular study. Other organizations do not own the land but have close working relationships with farmers from whom they lease the test plot area. Either of these approaches can be successful. The key to a successful trial is the effectiveness of the communications between all of the people involved with the trial. Critical times and activities must be clearly understood by all of those who participate in any aspect of the trial or the activities on adjacent crops. This includes those responsible for irrigation, application of maintenance materials, application of other research materials, and, where applicable, harvest of crops that may be adjacent

to the test plot. If the nature and goals of the study are clearly understood by all of these people, the chance of errors that may compromise the quality and integrity of the trial will be minimized, and the chance of trial success will be maximized. Everyone must realize that if anything happens that may impact or compromise the quality or integrity of the study, the Study Director must be contacted immediately and apprised of the situation. This allows the Study Director access to the maximum number of possible solutions to the problem. Solutions may include termination of that particular trial and starting it again in another location during the same cropping season. Everyone must understand that delaying the delivery of bad news only makes the news worse and reduces the chances of successful correction of the situation.

4 Best practices in conducting field study

4.1 Protocol development

All of the previously described planning is necessary to understand clearly the goals and implications of all activities associated with the study. 40 CFR 160.120 outlines the specific items that must be covered in the protocol. These items are:

- general information [to include: descriptive title of the study, statement of purpose of the study, name and address of the sponsor, signature line for Study Director, signature line for Study Director's management, name and address of the testing facility(ies), proposed experimental start date, proposed experimental termination date, proposed statistical methods, records to be maintained, instructions for GLP Compliance Statement is included]
- description of the test system [to include: crop species, source of supply, method of identification, justification for selection (e.g., EPA guidelines, proposed application crop/soil type)]
- test and (if applicable) control substances identification by name, Chemical Abstracts Service (CAS) Registry number, and/or code number; route of administration/application; reason for the choice of the route of administration; solvents and/or other materials used to solubilize or suspend the test (or control) substance before mixing with carrier; surfactant type and rate specified, if required
- methods (to include: description of the experimental design, methods for the control of bias, dosage levels, method and frequency of dosage administration)
- feed and water (for plant studies irrigation and fertilization) (to include: identification of the water source, specifications for levels of contaminants).

The most critical information in preparing a protocol that will ensure the success of the field residue trial involves:

- test substance to be tested (formulation type, strength, any storage constraints, any special handling requirements, etc.)
- test system (crop to be treated), to meet this requirement, the protocol will have to be specific enough to cover the items critical to the study but open enough to allow for local practice to be followed in the conduct of each trial; this becomes particularly important when dealing with many of the fruit and vegetable crops where unique

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local practices are necessary to ensure a successful crop (e.g., bedding and staking of plants in one production region as opposed to row crop production practices of this same crop in another production region), and, since the practices to raise a crop vary from region to region within the USA, allowance must be made to accommodate these practices in the study protocol
- whether or not adjuvants will be required as part of the spray solution
- method of application (do not make this so restrictive that local practices cannot be used)
- the use rate to be applied (if multiple rates are to be applied, the timing and sequence of each rate must be listed)
- application time (including the interval between or specific crop stage time of each application if multiple applications are to be made)
- PHI for the crop (for early season applications this may need to be estimated in the protocol and then confirmed upon harvest of the crop; for applications close to the harvest time for a specific RAC, the PHI must be in specific days/hours after the last application and be clearly described in the study protocol)
- harvest time anticipated for each of the RACs (e.g., will harvest be at normal maturity and staging for the RAC, or will the harvest be early/late, etc.)
- date the trial analytical results will be required (this will dictate when the RAC samples must be available from the field)
- any unique or unusual requirements that will be necessary to obtain the necessary RACs to ensure the maximum use of study data to support the desired marketing license.

Once the above information is available, the field residue protocol can be written. Development of protocol templates can easily ensure that all of these requirements are covered in the protocol in a consistent and uniform manner. Once a draft of the protocol has been prepared, all members of the planning team should review the draft for accuracy, completeness, and clarity. The team should assure that the GAP is clearly represented to confirm that the study will meet both marketing and registration goals. Once suggestions from the study planning team have been incorporated into the draft, the protocol can be forwarded to quality assurance (QA). The QA audit will assure that GLP is covered and that the purpose of the study is clear to someone who was not involved in the planning of the study. Suggestions from QA can then be incorporated into the final draft of the protocol that is then ready for management and Study Director signatures. Once the Study Director has signed the protocol, the study can be initiated at any time. If this planning and protocol preparation process has been successful, the stage is set for a successful field residue study which will be completed in a timely and cost-efficient manner. A study so designed will provide realistic residue levels from which proper residue tolerances can be established.

4.2 The test site

4.2.1 Site preparation

Preparation of a site for field residue testing should follow the same procedures as for standard agricultural production for the crop in question. If a pesticide is intended to introduce new agronomic or horticultural practices, then these practices should be

followed in preparing the test site for the field residue study. The type of tillage, the timing of crop production activities during the growing season, and other practices specific to the test system should all be according to local practices in order to help ensure a representative crop and RAC sample at the end of the trial. Irrigation prior to planting a crop, adequate pruning, and winter/spring treatments of a tree crop are examples of things that must be considered in preparing the site for the field residue trial. If the standard site preparation practices for a commercial crop are followed in preparing a site for a field residue trial, the trial will stand the highest chance of being successful.

4.2.2 Test location selection criteria

The trial sites must be located according to the guide in Table 5 of 860.1500. For most studies, the selection of the test site is not a critical problem so long as the site is located in a major production region for the crop under consideration. Since the RAC to be analyzed is intended to represent commercial production, the site from which the RAC will be harvested must also be representative. However, there are important considerations that do need to be taken into account in selecting the actual location of the trial. The ability of the PI to manage the study is probably the most important consideration. Having ready access and the ability to control access to the site will provide maximum convenience for the PI conducting a field residue trial. Being able to maintain environmental conditions at the site during the testing period will ensure that drought, wind, or flooding will not negatively impact a trial (e.g., irrigation, windbreaks, and drainage are important site selection considerations). Being able to ship samples directly from the test sites or to move samples from the test site to freezers will help ensure that sample integrity is maintained after harvest. The ability to control pests during the production season will help ensure that high-quality samples are harvested in a timely manner for the trial. Although PI-owned research farms are the easiest way to meet these requirements, remote sites can also be used as long as appropriate accommodations to the unique needs of the site relative to these study critical issues are addressed.

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Using land that has been in standard crop production helps to ensure a successful trial. A few site selection choices that could easily complicate the successful conduct of a trial are:

- a site that has been idle for an extended period of time
- land that may have been abandoned or is in the process of being reclaimed
- a site that has recently been disturbed (e.g., on top of a new tile drain or a utility easement, or following a flood, etc.).

The PI must be judicious in the selection of the test site in order to maximize the chance of a successful trial and in meeting the study objectives.

4.2.3 Test site information (soil, water, weather, slope, wind, history)

The type of field residue study being conducted will determine the amount of test site information required and the rigor required to obtain this information.

For studies involving test substance application to soil, there may be a requirement for more soil information than for studies where applications are made to foliage of established crops. The study protocol should describe any specific requirements relative to soil type selection and how to confirm the soil characteristics for the study. Most studies simply require that the soil be identified by its name (e.g., Keystone silt loam) and composition (e.g., percent sand, silt, and clay). This information can typically be acquired from farm records, a soil survey of the local area, or a typical soil analysis by a local soil analysis laboratory. In some instances, a GLP compliant soil analysis must be completed. The study protocol must clearly define what is needed and how it is to be obtained. Unless specified in the protocol, non-GLP sources are adequate to identify the soil and its characteristics. The source of the soil information should be identified in the field trial record.

Pesticides used on crops grown on the test site in previous seasons may also have an impact on the outcome of a field residue trial. Carryover of prior pesticide applications could contaminate samples in a new trial, complicate the growth of the crop in a trial, or cause interference with procedures in the analytical laboratory. For this reason, an accurate history of what has transpired at the potential test site must be obtained before the trial is actually installed. The protocol should identify any chemicals of concern. If questions arise when the history is obtained, they should be reviewed with the Study Director prior to proceeding with the test site. In most annual crop trials, this will not be a significant issue owing to crop rotations in the normal production practices, because the use of short residual pesticides and different chemical classes is often required for each respective crop in the rotation. However, in many perennial crops (tree, vines, alfalfa, etc.) and monoculture row crops (cotton, sugarcane, etc.), the crop pesticide history will play a significant role in trial site selection.

Another important test location factor is the availability of water for irrigation and for preparation of the spray solution. The use of culinary water sources (either private or public water sources intended for human consumption) or groundwater (from wells) is usually less problematic than using water from surface sources (rivers, lakes, or canals). If surface water is used for the study, care must be taken to ensure that farm production activities upstream from the plot area have not contaminated the water supply with pesticides that could contaminate the plot area. Careful site selection will help avoid problems from the water available at the site.

The slope of the land upon which the field trials will be established and the direction of the prevailing wind must be taken into consideration when locating the treated and untreated plots in a field trial. The protocol may specify a certain separation distance for the plots; however, the PI must ensure that the plots are located with adequate separation to prevent contamination of the untreated plot during the course of the trial. The untreated plot must be located up-slope and up-wind from the treated plot to reduce contamination from wind or rain. When the land is level or the wind is not from a reasonably constant direction, then distance may be the only feasible way to ensure that plot integrity is maintained. Careful attention to plot placement in the field and documentation of this location in the field notebook will help minimize questions or concerns about the trial site during preparation of the final report.

4.2.4 Field notebooks and other test site information (labels, shipping papers, etc.)

Record keeping is as critical to the success of a field residue trial as the actual application and sampling activities in the trial. If key activities (test system definition, application, sampling, etc.) are not adequately documented, the trial may not qualify to be used in the final report and for the tolerance-setting purpose. Other activities (cropping history, soil characteristics, weather information, etc.), although less critical to trial success, may also compromise the value of the data collected during the field trial. Field notebooks should provide a place to record all of the information that would be necessary to reconstruct a study. The field notebook may consist of either very detailed notebooks or simple study forms provided to the PI by the Study Director. Alternatively, the PI may be instructed to create a record on their own to cover the items specified from a list provided by the Study Director. Whichever way the Study Director desires to have the field information recorded will be adequate provided that the PI is diligent and keeps the record current as each activity is completed during the test period. Some of critical items that need to be recorded include:

- a copy of the protocol and either an index or actual copy of standard operating procedures (SOPs) to be followed
- a listing of all of the personnel involved in the trial and a place for each to sign a statement of authenticity and GLP compliance
- a chain of custody (COC) of the field notebook or trial record
- a compilation of protocol, SOP, or GLP deviations
- a communication log (telephone, mail, fax, and e-mail)
- test substance information (COC, receipt, use, and final disposition log)
- test site information [address, soil type, slope, history (to include crop, fertilizer, and pesticide history as required by the protocol), plot dimensions and location relative to permanent markers, test system preparation and maintenance, etc.]
- application records [equipment description, calculations relative application plan (amount of test substance to weigh out, amount of spray volume to prepare, speed to travel through the plot, width of application pattern, etc.), calibration of equipment to verify ability to meet application plan, verification of actual application (actual amount of test substance weighed out, actual volume of spray solution prepared, actual delivery rate, actual time spraying the plot area, etc.), application conditions (temperature, humidity, wind speed, time of first rain after application, etc.), and source of water used to make the spray solution, etc.]
- sample collection and storage information (how the samples were harvested and sampled (actual sampling PHI, actual activities or SOPs followed), what was actually sampled, weight or number of items sampled as appropriate, time between sampling and freezing, etc.)
- shipping information [including complete identity of what was shipped, how it was shipped, to whom it was shipped, shipping condition (frozen or ambient), date of shipment, COC to be completed upon receipt at the receiving laboratory, etc.]
- meteorological information [location of weather station relative to the test plot; dates of rainfall and/or irrigation; daily record of maximum, minimum, and mean temperatures; unusual events (hurricane) or conditions (drought) and how they affected the growth of the crop and samples derived therefrom]

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- a place to record any other data or information the Study Director may require (e.g., index to SOPs, training records, CV for PI and trial personnel, maintenance log, temperature logs, and other facility records that may be necessary to confirm the validity of the trial).

The form or format of the notebook is not as critical from a GLP compliance standpoint as the completion of the record in an accurate, timely, readable, and attributable manner. Company and PI conventions typically have evolved into cost-effective and very efficient data notebooks for field residue trials. These notebooks contain the actual raw data for the trial and once begun become extremely valuable legal parts of the study record. The notebooks should be audited by QA during the field phase of the study as well as at the end of the trial before the notebook is returned to the sponsor organization. The quality of the trial is easily reflected in the quality of the field notebook at the end of the season.

4.2.5 Critical site/weather information

The protocol and the field notebook will typically define weather information that will be critical to the interpretation of study results. Temperature, irrigation, rainfall, wind, cloud cover, and relative humidity can all have an impact on the growth of crops, development of pests, and performance of pesticides. The study team must clearly identify any and all of these items which may impact the outcome of a particular study. The items so identified and defined must then be clearly listed in the protocol and the field notebook along with preferred ways to manage or control them. Such instructions as 'do not apply if rain is anticipated within 2 h following the application' provide valuable guidance to the PI. Typically the wind speed and direction, temperature, relative humidity, and cloud cover should be recorded at the time of the application. The time between the application and the first rainfall is another important weather item that typically is to be recorded following each application. If unusual weather events appear eminent, the Study Director should be contacted, and the possible impact of these events on the study should be discussed. Preparation for a hurricane or a frost may seem like something that would be impossible to adjust to, but often with pre-warning, the Study Director can suggest ways to minimize the impact of these potentially damaging weather events on the trial. The key is to communicate openly and quickly when events begin to develop.

4.3 Test material

4.3.1 Test material characterization and Certificate of Analysis (COA)

The test substance used in a field residue study must be clearly defined and properly identified to ensure that the correct chemicals are used for the study. This process is called test substance characterization (40 CFR 160.105). The characterization of a test substance includes confirming the test substance is what was intended and that the test substance represents the actual commercial product that will be marketed. The test substance may be acquired from either a commercial production run or from

a special laboratory preparation. Whatever the source, the test substance must have a known formula, a known list of ingredients, and the actual percentage of a.i. to be used in the commercial product that will be registered by the EPA. An analytical assay of the test substance must be made to confirm that the strength is within the nominal range to be registered for the product. The results from this assay are used to prepare a COA which confirms the suitability of the test substance for use in the study. In addition to knowing that the correct test substance has been prepared, the test substance must be stable during the period for the study from preparation until use [40 CFR 160.105(e)]. The stability of the test substance may be measured after frozen storage or after accelerated aging at elevated temperatures. The stability of the test substance at the elevated temperatures bears a direct correlation to the time the test substance may be stored at ambient conditions. Requirements to store at median temperature may result from the accelerated aging study. The spray solution homogeneity and stability over the period of time required for the application should be known [40 CFR 160.113(a)(1)]. Test substance characterization is a vital part of the field residue study. Characterization must be completed in a timely fashion, accurately documented, and clearly reported in the study record if the study is to be successful.

The chemical hazard class must be determined for all pesticides before they are shipped in the USA (49 CFR). This regulation also describes the packaging, marking, labeling, and condition for shipment which must be met for air, water, rail, or truck transport. Chemical handlers and packers must be specifically trained and registered with the Agency in all aspects of shipping and handling components of test substance offered for transportation within the USA. In most instances, the Material Safety Data Sheet (MSDS) must be included with the test substance when it is shipped to the PI. These requirements have taken on added importance in recent times and must be strictly followed in order to prevent severe legal penalties for non-compliance with Department of Transportation (DOT) regulations. The PI should also be supplied with a copy of the MSDS prior to initiation of the study so that adequate safeguards can be implemented before critical study phases are executed.

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4.3.2 Chain of custody (COC)

The movement of the test substance during the course of a field residue study must be tracked to assure that the integrity of the test substance is maintained [40 CFR 160.185(a)(10)]. The COC can be accomplished in a number of ways. In the simplest situation, every person signs their name on a piece of paper that accompanies the test substance when they handle the test substance. Eventually the COC will list the names of all those who handled the test substance during the course of the study. Shipment, receipt, weighing, and final disposition of the test substance container must all be tracked and promptly recorded if an unbroken COC is to be present at the end of the trial. The completed COC becomes an essential part of the field residue trial record.

4.3.3 Storage and disposition requirements

Any unique storage requirements, if they exist, must be supplied with the test substance when the test substance arrives at the testing facility. Most test substances for field

residue trials can be conveniently stored under ambient conditions. No matter what the technical storage requirements may be for the test substance, the temperature of the storage conditions must be monitored and recorded in the trial record.

The Study Director will determine how the test substance may be used following the last application in the trial. Under US GLP regulation, the test substance container must be retained until the completion of the study [40 CFR 160.105(c)]. For residue trials that involve a commercial product, the Study Director may allow use of the remaining test substance in other crop production activities. For research products, the Study Director may allow use in other research trials. If either of these options is allowed, the amount of test substance removed from the test substance container is recorded in the test substance log along with where the test substance was used. If these options are not allowed, the test substance and the test substance container should be prepared for shipment and returned to the Study Director for storage until the completion of the study. When the test substance is shipped anywhere in the United States, appropriate DOT requirements must be followed. The PI will need to obtain the instruction for shipment from the sponsoring organization. The COC will be concluded when the Study Director or the agent of the Study Director signs for receipt of the container and any remaining test substance are placed in final storage until the completion of the study.

4.4 Application phase

Application of the test substance to the test system is without doubt the most critical step of the residue field trial. 'Under-application' may be corrected, if possible and if approved by the Study Director, by making a follow-up application if the error becomes known shortly after the application has been made. 'Over-application' errors can usually only be corrected by starting the trial again. The Study Director must be contacted as soon as an error of this nature is detected. Immediate communication allows for the most feasible options to be considered in resolving the error. If application errors are not detected at the time of the application, the samples from such a trial can easily become the source of undesirable variability when the final analysis results are known. Because the application is critical, the PI must calculate and verify the data that will constitute the application information for the trial. If the test substance weight, the spray volume, the delivery rate, the size of the plot, and the travel speed for the application are carefully determined and then validated prior to the application, problems will seldom arise. With the advent of new tools such as computers and hand-held calculators, the errors traditionally associated with applications to small plot trials should be minimized in the future. The following paragraphs outline some of the important considerations for each of the phases of the application.

4.4.1 Calculation/preparation of application solutions

There are many ways to determine the weights and volumes to use in an application to a residue field trial. If calculated correctly, all of these methods are adequate. No

matter what method of calculation is used, the following must be determined:

- amount of test substance to weigh out
- the total spray volume to prepare (include any surfactants or other adjuvants in this number)
- the delivery rate of the sprayer (a combination of nozzle type and spray pressure)
- the actual area to be treated
- the travel time that the application will take.

For small plot work, the number of significant digits used in these calculations must be considered in order to be accurate enough for the testing involved. Typically, two or three significant digits will be adequate; however, either the protocol or the facility and sponsor SOPs should define the accuracy required. Once these calculations have been made, they should be verified in an independent manner to ensure that a successful application will be made. Again, the use of computer programs or pre-programmed hand-held calculators easily facilitate this procedure.

4.4.2 Calibrations of application equipment

The equipment to be used in the application of the test substance is usually used for many trials each utilizing independent application settings. Therefore, before an application can be made, the equipment must be calibrated and adjusted to confirm that the equipment is ready to make the application. Calibration runs (minimum of three independent runs) should verify that the system is operating consistently, uniformly, and as expected. These runs must measure both for the delivery volume of the sprayer and the travel speed of the application equipment (tractor, hand-held boom, etc.). If the test substance changes the viscosity of the spray solution very much, a placebo spray solution that closely mimics the intended spray solution may be needed to calibrate the sprayer accurately. Also, the soil surface on which the speed calibration is made should be comparable to the soil surface of the plot area. If the speed calibration is made on a hard flat surface when the plot area is soft (e.g., recently tilled or irrigated plot area), then the chance of an 'over-application' being made is highly likely owing to a slower speed during the actual application. Conversely, the chance of an 'under-application' will exist if the surface of the plot area allows for faster travel time during the application. Attention to these details will greatly reduce the chance of problems in the application due to poor equipment performance at critical times. The calibration activities must be accurately recorded in case concerns relative to the application arise at a later date. This record can be critical in determining the possible resolution of questions or concerns that may arise when preparing the final report. Close attention to detail and clear, immediate recording of activities cannot be overemphasized during these activities.

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4.4.3 Stability issues

At times, unexpected events delay application of the test substance after the spray solution has been prepared. Most test substance spray solutions are stable for a reasonable period of time. However, the protocol, SOPs, specific test substance guidance

documents, or the Study Director must be consulted if the application is delayed more than an hour or two. If the test substance does not make a spray solution that is stable for the duration of the delay, a new spray solution must be prepared. Since the amount of test substance is often limited, the standard practice of most PIs is to make certain everything is ready to make the application before actually adding the test substance to the spray diluent. If this practice is followed, problems presented by last-minute rainstorms, wind storms, travel problems, equipment problems, etc., are minimized.

4.4.4 Application phase QA components

Good Laboratory Practice Standards (GLPS) require that the QA unit audit each study at intervals adequate to ensure the integrity of each study [40 CFR 160.35(b)(3)]. The application of the test substance to the test system is one of the most critical activities in a field residue trial. The presence of a highly qualified and competent QA during the application of the test substance is a valuable way to assure the quality of the application. Often this independent observer can see something amiss and bring the problem to the attention of the PI. The early warning can help to correct potential errors before they are made. The QA should make an independent verification of the calculations and calibrations as they are made. If this is done, errors or oversights should become apparent to the PI in a timely fashion. Another important contribution of the QA at the application is the role as a witness of and an independent verification of the actual events of the application. This assurance to study management and to the Study Director is an important contribution of QA to the overall study quality.

4.5 Sampling phase

4.5.1 Type/size of crop samples

One of the great benefits of the new guidelines is that they remove all doubt as to what constitutes a sample in each crop to be tested. Table 1 in OPPTS 860.1000 identifies the actual RAC to be harvested from each crop. The footnotes in this table add considerable detail to the description of these samples and should be considered closely when preparing the protocol and defining the samples to be harvested. OPPTS 860.1500, pp. 80–82, define the size of the RAC samples to be collected for analysis from each crop. Some samples are defined simply in terms of either the weight or the number of commodity to harvest. Other samples are defined as a combination of these two measures (e.g., 24 fruits, 12 if large, for a minimum sample size of 2 kg, etc.). OPPTS 860.1500 requires one sample from the untreated plot and two representative samples from the treated plot to be harvested. For large bulk samples, such as corn stalks or watermelons, the harvested sample units may be divided into smaller fractions such as thirds or quarters, and then one fraction from each sample unit is combined to form the final sample which represents the RAC. Usually the protocol or sampling SOP provides any necessary additional guidance relative to reducing the bulk of the samples. The intent of the sampling requirements of the protocol must be clearly understood, and the actual sampling procedure must be accurately

documented. If bulk reduction is done, extreme care must be taken to ensure against sample contamination during the process.

4.5.2 Sampling methods

Sampling can be as simple as picking fruit from a tree and digging potatoes from the ground or as complex as harvesting with a mechanical harvester. Samples should be harvested is such a way as to prevent bias in the samples (OPPTS 860.1500, p. 2).

Several sampling techniques are identified in most agricultural statistics books. The Study Director should specify the method to be used if there is a specific method to be followed. Often the harvesters simply have to collect samples from the plot in a random or nonsystematic way. Harvesting samples in a nonsystematic way ensures that each item in the plot stands an equal chance of being selected. Usually the only things to be avoided are the ends and edges of the plot. All other produce inside the plot area should then stand an equal chance of being included in a harvest of representative samples from the plots. If the harvest is done with a mechanical harvester (such as a small grain combine or a cotton picker), then nonsystematic removal of samples from the harvest stream (sometimes called grab samples) as the harvester progresses through the plot is an acceptable way to collect the necessary samples.

4.5.3 Residue decline study sample requirements

OPPTS 860.1500, p. 16, indicates that 3–5 sampling points should be included in the decline trials. For applications close to the normal harvest time, the RAC may be harvested at selected intervals between the time of final application and a normal harvest or slightly delayed harvest. If the application is made long before the normal harvest, then representative plant tissues (including immature RAC) may need to be harvested in order to stretch the harvest period. A single composite sample is all that is required from each selected time point, but two or more samples may be harvested to reduce uncertainty about the actual amount of residue present at each sample time interval. These decline samples should be collected and treated the same as normal RAC samples. The samples should be frozen as soon as possible after collection. The instructions for decline sample collection and handling described in the protocol should be followed closely.

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4.5.4 Processing study sample requirement

Processing studies require two types of samples, standard RAC samples and a sample for processing into the required processed commodities. The sample definition and size for the RAC samples are the same as for a standard field residue trial. The sample size for a processing sample is usually considerably larger than the RAC sample for the same crop. This may range from a few extra kilograms of RAC to nearly 1 t of produce for some of the extremely minor plant components (e.g., citrus oil). The processing laboratory responsible for sample processing must be consulted in setting the amount of RAC to be harvested for these samples. The processing sample size will be determined by the processing equipment's functional sample need to operate effectively.

The RAC samples harvested in a processing study should be frozen immediately upon harvest and handled exactly as other RAC samples are handled. However, as a rule, most samples to be processed must not be frozen prior to processing. Exceptions to this rule may exist for cereal grains or cotton gin by-product studies where the RAC is very dry and dormant at harvest. Freezing fresh market RACs (e.g., fresh fruits and vegetables) prior to processing would typically render the sample unsuitable for processing and would not allow the processing equipment to function properly. For convenience, the trials for a processing study should be located close to the processing laboratory to facilitate movement of the unfrozen processing samples from the field to the laboratory. Once the processed fractions have been generated, they should be frozen until analyzed. This preserves any residues that may be present. As an alternative to the RAC sample harvest in the field as described above for processing studies, the RAC samples may be collected from the bulk sample at the processor's laboratory. This has the added advantage of subjecting the RAC samples to the same conditions as the 'processed sample' prior to processing and may give more representative RAC samples than those harvested in the field separately from the processed sample. This approach may be preferable since the final analytical results may be more consistent between the RAC and the processed commodities.

4.5.5 Sample identification

Samples from field residue trials must be clearly identified. 40 CFR 160.130(c) indicates that the identification shall include the name of the test system, study number, nature of the sample, and date of collection. The identifying label for the sample must be located on the sample container in such a manner as to preclude error in recording data as the samples are handled and processed. The label must be legible, durable, and resistant to freezing conditions. The sample identity must be unique for each sample in a study to preclude confusion of samples during the analytical phase of the study. Sponsors have developed systems for sample identification and labeling that must be followed precisely to assure sample integrity throughout the study.

4.6 Sample storage and shipping

4.6.1 Storage requirements/conditions

The crop samples harvested at the end of a field residue trial are extremely valuable and must be treated with meticulous care to maintain their integrity until analysis is completed. If possible, samples should be placed in a freezer within a minimum of 30 min following harvest. If this is not possible, effort should be made to begin cooling the samples as quickly as possible after harvest. Cooling may be with blue-ice packs, crushed ice, or dry-ice depending on what is available to the PI and the distance to the field laboratory where the longer term storage will take place. If the transit to the laboratory will require several hours, dry-ice or the use of portable field freezers would be desired by most Study Directors. Cooling and freezing of the samples are essential to maintain the integrity of the samples and to ensure that unusual residue levels or metabolic by-products are not induced through a short period of overheating prior

to freezing. Most study plans will give specific instructions relative to the handling of samples after harvest and will indicate if there are unusual measures to be taken to ensure that the samples continue to be representative of the crop from which they were harvested.

4.6.2 Shipping options/documentation

Depending on the distance of the field location to the analytical laboratory, shipping may or may not be a problem. If the field is close to the laboratory, the samples may actually be delivered directly to the laboratory and frozen there. This is typically not the case, and some form of commercial shipment must be used. In the USA, an excellent infrastructure exists for either airfreight (typically Federal Express) or ground shipment [typically Accurate Cargo Delivery Systems (ACDS)] for frozen samples. Both of these commercial shippers have excellent records of on-time delivery for these very fragile and expensive samples. Air shipment requires the use of dry-ice to ensure that the samples remain frozen during shipment. Ground shipment is typically via GLP compliant freezer trucks. Depending on the timeliness of sample harvest or urgency of sample arrival at the analytical laboratory, one of these methods of shipment may be more efficient than the other. In most instances, ground shipment is more economical and convenient but takes more time. Air shipment is faster but more expensive and requires the use of dry-ice and close coordination of all participants in the shipping process (PI, shipper, delivery to the laboratory, and available personnel at the laboratory when the samples arrive).

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Whichever method of shipment is chosen, the samples must be packed in an appropriate shipping container to ensure that the journey to the laboratory goes without incident. These containers may be cardboard boxes or plastic ice chests. Different sponsors have found success with both of these containers and will typically provide what they prefer to be used. The samples must be logged as they are placed in the containers, and care must be taken to ensure that no opportunity for thawing or contamination occurs during the packing process. Typically, untreated samples are placed in one container, and treated samples are placed in a second container. However, if they are shipped in a single container, as may be the case for small samples, then, typically, a fixed divider is placed in the shipping container to keep the two samples from coming in direct contact with each other. Since these samples are so valuable and critical in the registration process, the marginal cost of an extra shipping container is usually not a reason to take a chance of contamination of the untreated samples, which could jeopardize the study results.

Once the samples are all in the appropriate shipping containers, the containers are closed and sealed shut. Shipping papers (sometimes called bill of lading) are then prepared and placed on or in the last box prior to being sealed. If desired, this paper can be prepared to serve as both a shipping log and as a formal chain of custody for the samples during shipment. If this process is followed, the shipping paper will list the study number, the analytical laboratory, the trial location that generated the samples, the date the samples were harvested (PHI) and sampled, the sample identity, a place for the shipper to sign as to the contents of the shipment, and a place for the receiving laboratory to sign upon receipt at the laboratory. A copy of this document would be retained by the shipper and included in the field notebook. The original

would become part of the laboratory record associated with the samples once they were logged into that facility.

4.6.3 Processing study samples

The RAC harvested for a processing study should be shipped or delivered to the processor as soon as possible following harvest. Even though the commodity to be processed is not to be frozen prior to processing, care should be taken to keep the commodity cool or from becoming overly heated. Cooling may be accomplished by placing the samples in the shade if the samples are to be held for only a short period. Alternatively, the samples may be placed in a refrigerated storage area for longer storage times. Care should also be taken to keep the samples from becoming desiccated by direct exposure to high temperatures, wind, sunlight, etc. If samples are to be transported in open vehicles at highway speed, containers that will minimize the potential for heating or drying during transit should be used. The RAC for processing studies must be kept as fresh as possible until processing can be completed. The sample processed must be representative of the produce which is subjected to commercial production and processing operations. With appropriate care and attention, the commodities created during processing activities will be representative of commodities commonly found in commerce.

RAC samples from a processing study should be handled exactly as RAC samples from a field residue trial. They should be frozen as soon as possible following collection. Once the processing commodities have been created, they should be frozen and shipped to the analytical lab as quickly as possible. Both the RAC samples and processed samples from a processing study must remain frozen throughout the shipment and storage period of the study in order to preserve residue integrity.

4.6.4 Storage stability

The integrity of the pesticide residue within the RAC samples over time is a critical component of the tolerance setting process. Ideally, one would like to harvest samples and immediately analyze them for potential pesticide residues. However, since this is not practical in most situations, OPPTS 860.1380 outlines the procedure to follow to be able to demonstrate RAC sample and pesticide residue integrity over the time that samples are stored frozen. If samples can be analyzed soon after harvest (30 days or less), a storage stability study may not be required. Since this is seldom the case, most registration programs require storage stability data. Although there are several approaches to these studies, the most realistic approach is to integrate this study into the RAC studies as they are conducted for a product. Additional sample sizes may be required for this study, especially if the samples are to be stored for a long period of time before analysis. The results from this study will be most representative if the study is conducted on control plants that have been weathered and aged prior to being harvested exactly like the RAC samples used for the residue studies. Alternatively, crops from a known source could be selected and used for the storage stability study. If this alternative procedure is followed, extra effort will be required to identify the crop history and to validate the samples to be used for the storage stability study.

4.7 Sample preparation

4.7.1 Sample homogenization

To facilitate modern analytical methods, the sample must be homogenized or macerated such that aliquots can be removed for analysis. This homogenization must be done in such a way that the sample integrity is not compromised. This usually requires that the samples be homogenized in a frozen state often by the use of dry-ice or other materials that will not allow the samples to thaw. If the samples must be thawed, they should be homogenized quickly and refrozen to prevent metabolism or decomposition of the residues during this short time. If this is necessary, this procedure should be completed as close to the time the samples will be extracted and analyzed as possible. Specific procedure and processing methods should be covered in SOPs that address the special needs of any particular RAC or fractions from a RAC. This is a critical part of the study and must be completed with extreme care to ensure that sample integrity is maintained. Extreme care must be taken to ensure that equipment used for these activities is maintained and cleaned appropriately to prevent contamination. SOPs will typically indicate that untreated samples are to be homogenized first followed by the samples expected to have the least residue and finally by the samples expected to have the highest residue level. Contamination of samples at this stage of the process will typically render a study unacceptable and may create issues that prevent proceeding with a registration. Following established laboratory procedures will ensure that the sample integrity is maintained throughout analytical phase of the study.

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4.7.2 Storage stability

OPPTS 860.1380 outlines the requirement concerning storage of residue samples. Data must be obtained that validates the stability or the rate of decomposition of the total toxic residue (TTR) in the RAC samples and any processed commodity between the time of harvest and the final analysis of the residue(s) in the samples. In an ideal world, the samples would be analyzed immediately after harvest or sampling. However, this is impractical and would not allow the efficient use of analytical equipment. Since RACs are harvested and sampled over a considerable period of time simply owing to the various crop maturity timings in the many cropping regions of a study, RACs from the trials will not all become available at exactly the same time. Therefore, an analytical sample storage stability strategy should be built into each registration project during the design phase of the studies. Storage stability data will typically be required for all magnitude of residue studies (crop field trials or processing trials). Several other important instructions are provided by this guide relative to setting up the storage stability portion of the study, containers to use, number and frequency of samplings, weathered field samples vs laboratory spiked samples, etc. This guide should be reviewed closely in designing the stability study, collecting the data, and reporting the results. The inherent stability of the residue will, to a large extent, determine the complexity of this portion of the study. If the residues are extremely stable, this study will be much simpler than if the residues decline or degrade over time. Extreme care must be taken to ensure that this study is done correctly to avoid serious review delays or actual rejection of the registration application.

4.7.3 Subsampling requirements

Subsampling for the standard assay samples and the storage stability samples must be done in such a way as to avoid prejudice of the results in any way. The techniques involved should be done in a way that does not introduce bias (e.g., sampling from a single place in the sample), diminish the representative nature of the sample (only taking from the edge or top of a sample container, etc.), or allow sample contamination during the process. Most organizations have established definitive SOPs for this sensitive task. Strict adherence to these SOPs is critical if the quality of the study is to be maintained at this stage of the testing process.

4.8 Field QA components

4.8.1 Critical phase

The GLP requirements for a field residue study indicate that each study be audited as needed to ensure the quality and integrity of the study (40 CFR 160.35). For this purpose, a study is divided into an in-life phase which includes all of the activities which involve the generation of samples to be analyzed and the analytical phase which includes analysis of the samples in the laboratory. The study may minimally be defined as the activities that occur between the application of the test substance in the field plot through the collection of data from the analytical instrument in the laboratory. The time period for the critical phase of a field residue study can be as short as a few weeks for a simple RAC study (e.g., a late-season application to a small number of trials on a crop that matures in a close interval over the whole production region). The critical phase may extend to 2 years or more for a field crop with a long crop production cycle (sugar cane, citrus, etc.). The guidelines indicate that the QA audits must be conducted at such times and intervals as to ensure the Study Director and management that the study is progressing as planned and that all aspects of the study are under control [40 CFR 160.35(b)(3)]. For short studies, this usually means that one or two in-life audits (typically an application and/or a sampling activity) plus a facility and records audit will be adequate. For a longer term study, such as a field crop rotation study, conducting audits on a time interval basis (such as every trimester or at 6-month intervals of the in-life phase of the study) may be needed.

To assure independence and unbiased auditing, trained QA individuals must perform audits. QA auditors should not be involved in study conduct, and must be independent from study management [40 CFR 160.35(a)]. These audits may be conducted by the sponsor's quality assurance unit (QAU) or via a contract QA who will report back to the sponsoring organization. Any findings during an audit that are likely to affect the integrity of the study must be brought to the attention of the Study Director and management immediately [40 CFR 160.35(b)(3)].

In-life or critical phase audits must be completed in a timely and efficient manner. They must not detract from the conduct of the study or interfere with the execution of critical activities within the study. However, QA must be able to clearly determine the actual progress of the study. Audit reports must clearly identify the actual findings of the audit. The reports must be relayed to the Study Director and to study management in a timely manner. If deviations occur or if minor findings are reported, they must

be addressed in a manner that corrects or upgrades the issues as they relate to the study and/or the facility. The audit is an essential component of the trial and must be given adequate time and resource to ensure not only compliance but also improved performance over time.

4.8.2 Facility and record audits

The qualification of the facility to conduct a study is based on the quality standard and expectation of the sponsoring organization. Most organizations require a facility audit prior to contracting a study either in-house or with a contract organization. During this audit, the organization's overall compliance with GLP standards as well as their technical capability and capacity to conduct the field residue trials will be assessed. A PI may be an extremely competent businessperson or scientist but may not qualify to do a field residue trial if the necessary GLP training and documentation is not in place. Organizational charts, training records, job descriptions, SOPs, maintenance records, facility and personnel capabilities, and organizational effectiveness must all be considered during the facility audit. Since sponsors vary in their implementation of GLPs, there is a certain amount of latitude and variation in the contracting organizations as well. This leads to close relationships between certain sponsors and contracting organizations. The purpose of the facility audit is to ensure that the sponsor's expectations can be met and that the expectations are consistent with the GLP guidelines. One approach is not necessarily more acceptable than another; each approach simply requires a different level or type of oversight. If the philosophies of the sponsor and the contract organization are similar, the facility audit will indicate a good likelihood of study success.

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4.8.3 Audit communication

Audit reports that include findings and responses from the PI must be shared with the Study Director and management in a timely manner following the audit. If there are findings that may jeopardize the quality or integrity of the study, they must be reported to the Study Director immediately [40CFR 160.35(b)(3)]. These communications may be via documented telephone conversations, via written reports that can be mailed or faxed, or via e-mail as the Study Director determines. The nature of the findings will determine the speed at which the information must be made available to the Study Director. Audits with no finding or minor findings may be reported within a few days or a couple of weeks, if necessary, to allow the PI to complete a response to the finding. Serious issues (such as protocol, SOP, or GLP deviations) need immediate Study Director attention. Every effort should be made to inform the Study Director as soon as possible of the nature and potential impact of serious findings. In this instance, direct and immediate telephone or e-mail communication may be necessary. Once the audit reports have been reviewed and any findings have been addressed by the PI, the Study Director, and management, the formal QA audit report should be archived in the QAU audit archive. Corrective action, if necessary, should be recorded in the study record. Deviations should be clearly documented in the field notebook as well as any corrective action that was taken. The Study Director must assess the impact that the deviations may have on the study and record this assessment in the study

record. The deviation and corrective action become part of the final study record. QA audit reports are not part of the study record, but they must be maintained in a QAU archive should they need to be referenced at some reasonable time in the future.

4.9 Data presentation and communication

In 1986, the EPA published a 'Pesticide Registration Notice' (PR86-5) which outlines the format and structure of the report to be used in a pesticide registration submission to the Agency.[10] Section (h) of OPPTS 860.1500 outlines the requirements for reporting results from field residue trials. This guide outlines a reporting process that is compatible with the Agency's review process. The format suggested in the guide is not mandatory; however, all of the items suggested in the guide must be covered if the study report is to be successfully reviewed at the Agency. Since the 860 series guidelines were published, EPA and the Canadian Pest Management Regulatory Agency (PMRA) have collaborated to develop study report and review guidelines and templates. These templates and guidelines are consistent with the North America Free Trade Agreement (NAFTA) and the Organization for Economic Cooperation and Development (OECD) guidelines for international regulatory harmonization. Although these guidelines are still under development, they have been used successfully by both the EPA and PMRA to improve the timeliness of the review process. The status of these guidelines and examples of current templates can be found on the web sites for each Agency.[11–13] Working closely with the Agency prior to preparing a report will assure that the current report format is known and available to the petitioner in a timely manner. Following this format will ensure that upon submission reports are complete, accurate, and formatted in a way that will allow timely review by the Agency.

The Agency has recently published the 'Cross-Media Electronic Reporting and Record-Keeping Rule' (CROMERRR), which if implemented will govern how electronic data are managed and how electronic reports are submitted to the Agency.[14] The purpose of this rule is to reduce and eliminate obstacles to electronic record keeping and reporting across all EPA program offices. This rule is currently under review and out for public comment. Once the public comment has been considered and the review completed, CROMERRR will be published as a guideline and rule for data management and submission to the EPA. When enacted into law, this rule will establish requirements that assure equivalency between electronic records and paper records for all reports going to the Agency. CROMERRR will be EPA's counterpart to FDA's 21 CFR Part 11 that governs electronic record collection, management, archiving, and reporting. Once enacted, CROMERRR will impact record keeping and reporting procedures surrounding GLP studies and other reports submitted to the EPA. More information about CROMERRR can be found at http://www.epa.gov/cdx/cromerr_rule.pdf.

4.9.1 Field and electronic notebooks

In 1989, the field portion of residue chemistry studies began to be regulated under the EPA's Good Laboratory Practice Standard (GLPS) (40 CFR Part 160). At that time the only feasible means of collecting and reporting field data was via paper. Each sponsor

organization developed their own method of record keeping and reporting for field residue studies. All of these methods of study documentation were deemed acceptable by the Agency so long as the record was attributable, legible, contemporaneous, original, accurate, complete, and fully auditable. When these data quality issues are met, field study reports are easily prepared.

In recent years, with the advent of laptop, pad, and handheld computers, new electronic field notebooks have begun to emerge. Although these systems offer certain convenience for reporting data from field residue studies, there is considerable debate within the industry (both between sponsors and among PIs) concerning the field practicality and GLP compliance issues with implementation of these systems. Issues concerning system validation, data quality and integrity, contemporaneous data, original raw data, data processing, and archiving continue to be a source of considerable debate concerning these electronic notebook programs. In some business models, there is still no clear signal that there is an economic advantage to using the electronic field notebook over paper, while other business models declare significant savings when the electronic notebooks are used. Many field researchers still prefer the use of paper notebooks owing to their greater flexibility, adaptability, ease of use, cost, and low maintenance. Other researchers indicate that the electronic notebooks have brought excellent discipline and efficiency to their operations. The hardware and software associated with the various electronic notebooks are still under development and test, as indicated by significant upgrades and training requirements for users at the beginning of each field season. According to some sponsors and field researchers, the use of these tools, as they currently exist, adds considerable cost (either real dollars or additional time to enter the data into the electronic notebooks) either to the PI directly or to the sponsor of the field residue study. Additionally, the impact of CROMERRR on these tools will have to be resolved before they can be fully accepted and implemented as an industry standard. In the interim, a convenient tool that is being used by several companies is the use of an electronic field summary report for each trial prepared by the PI and submitted to the sponsoring organization. With this process, the paper notebook forms the raw data for the study, and the field summary report is simply a convenient way to extract the data for the final report. The transition field summary report may be a word processing document or a spreadsheet program. Since these tools are simply transition tools used to get the raw data into a final report format, they will not fall under CROMERRR at this time.

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4.9.2 Field reports components

The records required for field residue study authentication are the same records that would be required to reconstruct the study. Although this total volume of information is a necessary part of the GLP study record, the field summary report is a small fraction of that record. The field summary report is simply the information the EPA reviewers wish to see as they consider the data and determine how well they represent the crop situation for which the pesticide tolerance is being requested. At the current time, the field summary report should contain the information requested on pp. 48 and 49 of OPPTS 860.1500. The summary report for each test site in a study will typically form an appendix in the final study report. This information must be accurately extracted from the raw data notebook or field record and must be audited by

QA to ensure that the final report accurately reflects the raw data. As the new data and report templates/formats are developed and approved via the international regulatory harmonization efforts, the requirements for field residue study reports may change. Careful attention to the Agency web pages will assure that the most effective methods of data collection and reporting are followed. This should facilitate data management processes for field PIs, sponsor organizations, and reviewers at the EPA. Close adherence to these guidelines should lead to faster data reviews, more successful studies, and faster access to the market place for new product registrations.

5 Summary

Pesticide registration in the USA continues to be a very intensive and regulated process under the jurisdiction of the EPA. The amount of pesticide residue remaining on food or feed items is a critical component of the human exposure/risk assessment during the registration and subsequent management of all pesticides used in the USA. Publication of new testing guidelines entitled 'Residue Chemistry Test Guidelines' in 1996 significantly impacted the way field residue studies are to be conducted.[2] Close adherence to these guidelines will simplify the conduct of field residue trials and help ensure that data collected from such trials meet regulatory requirements. Studies conducted in such a manner will meet with faster regulatory review and allow businesses to bring products to market in the shortest possible time with maximum access to the markets they wish to participate in. Disciplined attention to detail during the planning, implementation, and completion of field residue projects is necessary if studies are to be completed, reviewed, and accepted in a cost efficient and time effective manner.

References

1. Office of the Federal Register National Archives and Records Administration, 40 CFR Parts 150 to 189, 'Protection of Environment,' Revised as of July 1, 1999, US Government Printing Office, Washington, DC (1999).
2. United States Environmental Protection Agency, 'Prevention, Pesticides, and Toxic Substances,' 'Residue Chemistry Test Guidelines,' US Government Printing Office, Washington, DC (1996). Also available on the World Wide Web: **http://fedbbs.access.gpo.gov** and **http://www.epa.gov/docs/OPPTS-Harmonized/860_Residue_Chemistry_Test_Guidelines/**.
3. 'Food Quality Protection Act,' US Government Printing Office, Washington, DC (1996). Also available on the World Wide Web: **http://www.epa.gov.oppfead1/fqpa**.
4. G.M. Markle, J.J. Baron, and B.A. Schneider, 'Food and Feed Crops of the United States,' second edition, Meister, Willoughby, OH (1998).
5. J.H. Martin and W.H. Leonard, 'Principles of Field Crop Production,' Macmillan, New York (1967).
6. N.F. Childers, J.R. Morris, and G.S. Sibbett, 'Modern Fruit Science, Orchard and Small Fruit Culture,' Horticultural Publications, Gainesville, FL (1995).
7. D.M. Considine and G.D. Considine, 'Foods and Food Production Encyclopedia,' Van Nostrand Reinhold, New York (1982).
8. United States Department of Commerce, Economics and Statistics Administration, Bureau of the Census, 'Agricultural Atlas of the United States,' in Volume 2, Subject Series, Part 1, "1992 Census of Agriculture," US Government Printing Office, Washington, DC (1995).

9. United States Department of Agriculture, National Agricultural Statistics Service, 'Agricultural Statistics,' United States Government Printing Office, Washington, DC (2000). Also available on the World Wide Web: **http://www.usda.gov/**, **http://www.usda.gov/nass**, and **http://www.mannlib.cornell.edu/reports**.
10. United States Environmental Protection Agency, 'Pesticide Registration (PR) Notice 86-5,' US Government Printing Office, Washington, DC. Also available on the World Wide Web: **http://www.epa.gov/PR_Notices/pr86-5.html.**
11. United States Environmental Protection Agency, 'Electronic Submission and Review Pilot Interim Guidance Documents,' US Government Printing Office, Washington, DC. Also available on the World Wide Web: **http://www.epa.gov/oppfead1/esr_pilots.htm**.
12. United States Environmental Protection Agency, 'Harmonization and Regulatory Coordination,' US Government Printing Office, Washington, DC. Also available on the World Wide Web: **http://www.epa.gov/oppfead1/international/harmonization.html**.
13. 'Harmonization of Environmental Chemistry and Fate Data Requirements Under NAFTA,' Regulatory Proposal PRO 2001-02, Health Canada, Pesticide Regulatory Management Agency. Also available on the World Wide Web: **http://www.hc-sc.gc.ca/pmra-arla/english/pdf/pro/pro2001-02-e.pdf**.
14. United States Environmental Protection Agency, Office of Environmental Information, 'Cross-Media Electronic Reporting and Record-Keeping Rule (CROMERRR),' *Federal Register* (40 CFR Parts 3, 51, *et al.*, Vol. 66, No. 170, pp. 46161–46195–FRL-7045-5). US Government Printing Office, Washington, DC. Also available on the World Wide Web: **http://frwebgate.access.gpo.gov/cgi-bin/getdoc.cgi?dbname=2001_register&docid=fr31au01-20.pdf**.

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Conducting crop residue field trials in Europe

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1 Introduction

Agriculture within the European region is diverse and can best be described by its diversity of crops, from the vineyards of France, to rice in Italy, and to the large expanse of glasshouses in The Netherlands. All of these situations require the use of approved pesticides to enable these crops to grow healthily in their situations.

Field residue data, which are generated to meet requirements in the pesticide registration process, are used to regulate the use of agriculture products within the European Union (EU). This article examines the best practices to conduct crop field trials and to generate crop residue samples in Europe in order to provide part of the data that the agrochemical producers of the active ingredients must provide to the EU Commission.

2 General issues and considerations in conducting residue studies in Europe

2.1 Regulation guidelines

European Union (EU) Council Directive 91/414/EEC on the authorization, use, and control of plant protection products, insecticides, fungicides, herbicides, etc., was adopted in 1991. This Directive set up a harmonized authorization system for the active ingredients (substances) used in plant protection products at the EU level. Member States may then approve products containing such EU-agreed ingredients for use in their territory. The 1991 rules make EU authorizations of active ingredients subject to a positive outcome of safety evaluations based on data provided by agrochemical producers. The target set for completing these evaluations was July 2003. An option to extend the July 2003 deadline for certain ingredients was also foreseen. Directive 91/414 also states that plant protection products authorized nationally can remain in use only until July 2003 or until a decision to authorize or ban them is reached at the EU level. When Directive 91/414 was issued, there were over 800 such ingredients authorized for use in the Member States.

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The progress made in such safety evaluations has been much slower than originally assumed. Of the more than 800 active ingredients to be evaluated by 2003, almost half remain to be evaluated at the Community level. If new strategies to improve the speed of review, such as improving the procedures for evaluating data, instituting tighter timelines, making better use of information technologies, and increasing personnel, are implemented as proposed by the Commission, the evaluation program may be completed by 2008. Proposals to amend Directive 91/414 and its timeline came forward in 2002. The deadline was extended to 2008, and withdrawal of active substances will be implemented in July 2003 if the proposals are not supported or have been withdrawn following a review of the dossiers.

2.2 *European comparable climatic zones/weather influences*

Europe is divided into two zones for the purpose of registration of new plant protection products within its community. The trial location should fall within these two distinct zones, namely the Northern and Central European Zone and the Southern European and Mediterranean zone. The climatic conditions and weather influences within each of the two regions described are assumed to be comparable. However, trial data should be representative of the areas where pesticide use is to be granted.

- Northern and Central European Zone
  This includes southern Sweden, southern Norway, southern Finland, Denmark, United Kingdom, Ireland, northern France, Belgium, The Netherlands, Luxembourg, Germany, *Poland*, *Czech Republic*, *Slovakia*, *Austria*, *Hungary*, and *Switzerland*.
- Southern European and Mediterranean Zone
  This includes Spain, Portugal, southern France, Italy, Greece, *Croatia*, *Serbia*, *Bosnia and Herzegovina*, *Slovenia*, *FYROM (Former Yugoslav Republic of Macedonia)*, *Turkey*, *Bulgaria*, *Romania*, and *Cyprus* (countries in italics are currently outside the EU).

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The separation of the two zones divides France into north and south, generally following the path of the Loire valley through the country. Data from different countries within the same region may reflect different cultural practices, and they may be rejected. The agricultural practice defining the worst-case situation should generally be used to generate the data used to define the maximum residue level (MRL). Results from noncomparable regions do not serve as a substitute for trials in the appropriate regions, but they do add to knowledge of the residue profile of the active ingredient. The evaluation of intended uses within the EU should be based on residue data mainly generated within the EU.

2.3 *Crop and grouping*

After the crops and the market viability of the product being registered have been assessed, the appropriate field trial sites need to be chosen. Selection of the trial site within the main growing regions for the specific crop type within each zone should

be made. The trials conducted should ideally be located in areas where crop usage is high. A majority of European countries produce their own data relating to the production and growth regions for each crop within a specific year. These data should be examined to select the major growing regions for the crop type.

By using crop groups, the total number of required residue trials may be reduced, and hence development costs of a new active ingredient may also be reduced. Extrapolation of residues from trials on the representative crops in a crop group may be used to support the use on the other crops within the crop group. The crops are divided into major and minor crops based on their consumption and area of crop grown together with their biological characteristics. Major crops generally form the strategic backbone of the registration of a new active ingredient and are outlined below (minor crops are italicized).

1. Fruits
   - (i) Citrus fruits
     Oranges, lemons, clementines, manderins, grapefruit, tangelos, tangerines
   - (ii) Tree nuts
     Walnuts, almonds, pecan nuts, sweet chestnuts, coconuts, *hazelnuts*
   - (iii) Pome fruit
     Apples, pears, *quinces, black chokeberry, mountain ash and medlar*
   - (iv) Stone fruit
     Apricots, cherries, peaches (including nectarines and similar hybrids), plums (*Prunus domestica* including all subspecies), c*ornel cherries*
   - (v) Berries and small fruit
     - (a) Grapes
       Table grapes, wine grapes
     - (b) Strawberries
     - (c) Cane fruit (other than wild)
       Blackberries, mulberries
     - (d) Other small fruits and berries (other than wild)
       Azarole, blueberries, buckthorn, cranberries, elderberries, gooseberries, rose hips, service berries
     - (e) Wild berries and wild fruit
       All crops
   - (vi) Miscellaneous fruit
     Kiwis
2. Vegetables
   - (i) Root and tuber vegetables
     Carrots, sugar beet, fodder beet, *beetroot, chicory roots, horseradish, Jerusalem artichoke, parsley roots, swedes*
   - (ii) Bulb vegetables
     Bulb onions, g*arlic*
   - (iii) Fruiting vegetables
     - (a) Solanacea
       Tomatoes, peppers
     - (b) Cucurbits – edible peel
       Cucumbers, *patisson (marrow), zucchini*

(c) Cucurbits – inedible peel
Melons
(d) Sweet corn

(iv) Brassica vegetables
(a) Flowering brassicas
Cauliflower
(b) Head brassicas
Brussels sprouts, head cabbage
(c) Leafy brassica
(d) Kohlrabi

(v) Leaf vegetables and fresh herbs
(a) Lettuce and similar
Lettuce, *cress, dandelion leaves, sarole*
(b) Spinach and similar
(Swiss) chard, leaves of beetroot, purslane
(c) Water cress
Water cress
(d) Witloof
Witloof
(e) Herbs
All crops

(vi) Legume vegetables (fresh)
Beans, green with pods, peas, green without pods

(vii) Stem vegetables
Leeks, *artichokes, rhubarb*

(viii) Fungi
(a) Mushrooms other than wild
Oyster mushroom, ring mushroom (Stropharia rugosoannulata)
(b) Wild mushrooms
All crops

3. Pulses
Beans, dry (including broad bean), peas, dry (including chick peas)
4. Oil seeds
Cotton seed, palm kernels, peanut, rapeseed, soya bean, sunflower, *hemp seed, gold of pleasure, linseed, mustard seed, pumpkin seed, safflower, soya beans*
5. Potatoes
Early and ware potatoes
6. Tea
Tea (*Camellia sinensis*)
7. Hops
Hops
8. Miscellaneous
Coffee, green, cocoa beans
9. Spices
All crops
10. Cereals
Barley, maize, oats, rice, rye, sorghum, triticale, wheat, *millet*

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11. Tea-like products
    All crops
12. Medicinal herbs and drugs
    All crops

3 Study planning phase

A specified number of residue trials must be conducted for each crop to provide sufficient data for each active ingredient.

3.1 Study objectives

In order to ensure that the desired information and data are generated**,** the study objectives must be clearly defined and understood by all parties involved in the conduct of the study. This is increasingly more vital when conducting multi-site residue studies covering a range of countries within the EU, all having their own native language. Based on the EEC Directive 91/414, the objective may be to support an EU new product registration or a country specific registration. Residue trials are most often conducted to:

- determine the maximum expected residue levels, which can then be used to determine the permissible maximum residue limit in or on plants (MRL)
- determine the most suitable pre-harvest interval (PHI) to minimise residues
- determine the behavior of residues in or on plants in relation to time (decline trial)
- determine the residues taken up from the soil by planting and growing succeeding rotational crops
- determine the transfer of residues into processed products (processing studies, e.g., purée, juice)
- determine the dissipation profile of residues in the air, soil, and water environment.

3.2 Role and responsibility of study personnel

3.2.1 Study Director

The Study Director must prepare a study plan and carry out the following duties:

- act as the single point of control for the overall conduct of the study and its final report
- approve the study plan and any amendments to the study plan by dated signature
- ensure that the quality assurance (QA) personnel have a copy of the study plan and any amendments in a timely manner and communicate effectively with the QA personnel during the study
- ensure that study plans and amendments and standard operating procedures are available to study personnel

- ensure that the study plan and the final report for a multi-site study identify and define the role of any Principal Investigators, any test facilities, and any test sites involved in the conduct of the study
- ensure the procedures specified in the study plan are followed, assess and document the impact of any deviations from the study plan on the quality and integrity of the study, take appropriate corrective action when necessary, and acknowledge deviations from standard operating procedures during the conduct of the study
- ensure that all raw data generated are fully documented and recorded
- ensure that computerized systems used in the study have been validated
- sign and date the final report to indicate acceptance of responsibility for the validity of the data and to indicate the extent to which the study complies with the principles of Good Laboratory Practice (GLP)
- ensure that after completion of the regulatory study, the study plan, the final report, raw data, and supporting material are archived.

3.2.2 Principal Investigator and study personnel

The Principal Investigator must ensure that the delegated phases of the study are conducted in accordance with the applicable principles of GLP.

The study personnel:

- must be knowledgeable in all pertinent principles of GLP
- must have access to the regulatory study plan and appropriate standard operating procedures applicable to their involvement in the study and must comply with the instructions given in these documents, documenting and communicating directly to the Study Director and/or, if appropriate, the Principal Investigator any deviations from the instructions
- must record raw data promptly and accurately and in compliance with the principles of GLP and be responsible for their quality
- must communicate any relevant known health or medical conditions in order to ensure the integrity of the study.

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3.2.3 Quality assurance personnel

The responsibilities of the QA personnel should include, but not be limited to, the following functions:

- maintain copies of all approved study plans and standard operating procedures in use in the test facility and have access to an up-to-date copy of the master schedule (overview of all studies being conducted)
- verify and document that the study plan contains the information required for compliance with the principles of GLP
- conduct inspections to determine if all studies are conducted in accordance with the principles of GLP and if study plans and standard operating procedures (SOPs) have been made available to study personnel and are being followed. Inspections can be study based, facility based, or process based, and records of all inspections should be retained

- review the final reports to confirm that the methods, procedures, and observations are accurately and completely described, and that the final report accurately and completely reflects the raw data of the regulatory study
- report promptly all inspection results in writing to management and to the Study Director, and to any Principal Investigator and their respective management, when applicable
- prepare and sign a statement, to be included with the final report, which specifies the types of inspections and their dates, including the phase of a study inspected and the dates that the inspection results were reported to management and the Study Director.

3.2.4 Facility management

The facility management has the following responsibilities:

- ensure that the principles of GLP are followed within its test facility and that a statement exists which identifies the individuals within a test facility who fulfil the management responsibilities
- ensure that a sufficient number of qualified personnel, appropriate facilities, equipment, and materials are available for the timely and proper conduct of regulatory studies
- ensure that a record of the qualifications, training, experience, and job description for each professional and technical individual is maintained
- ensure that personnel clearly understand the functions they are to perform and provide training for those functions
- ensure that appropriate and technically valid SOPs are established and followed and approve all original and revised SOPs
- ensure that a QA program is in place and is being performed in accordance with the principles of GLP
- ensure that for each study, an individual with the appropriate qualifications, training, and experience is designated as the Study Director before the study is initiated; replacement of a Study Director should be done according to established procedures and should be documented
- ensure, in the event of a multi-site study, that, if needed, a Principal Investigator is designated, who is appropriately trained and qualified to supervise any delegated phase of the study; replacement of the Principal Investigator should be done according to established procedures and should be documented
- ensure that the study plan is approved by the Study Director
- ensure that the Study Director has made the approved study plan available to the QA personnel
- ensure that a historical file of all standard operating procedures is maintained
- ensure that an individual is identified as responsible for the management of the archives
- ensure that a master schedule is maintained
- ensure that test facility supplies meet requirements appropriate to their use in a study

- ensure that clear lines of communication exist between the Study Director, Principal Investigator, QA program and personnel at all study sites
- ensure that test and reference items are appropriately characterized
- establish procedures to ensure that computerized systems are suitable for their intended purpose and are validated, operated, and maintained in accordance with the principles of GLP.

Individual test site management (if appointed) will have the responsibilities set out in items 2–6, 8, 11–14, 16, and 17.

A number of variations in working practices have developed in order to meet these regulatory requirements in the past few years. As an example, the Study Director may be based in the agrochemical sponsor organization, and the Principal Investigators are appointed in a specialized contractor organization and are only responsible for the conduct of their phase of the study, either field or analytical. Alternatively, the Study Director may reside in a contract organization where both the analytical phase and field phase of a study are being conducted. Here the contract organization may have its own field staff in various locations to act as Principal Investigators, or they may sub-contract the field phase or the analytical phase to another contractor. Whichever scenario is being followed, the Study Director has the ultimate responsibility to ensure the study is conducted successfully and all phases are in compliance with GLP.

3.3 Preparing the study plan

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For each regulatory study, a written plan must exist prior to initiation of the study. The study plan must be approved by dated signature of the Study Director and verified for GLP compliance by QA personnel.

The study plan should contain practical instructions concerning the conduct of the field study and at the same time allow flexibility to the field staff with regard to his freedom to react to unforeseen events. Normally study plans are written in English since most of the technical staff and regulators involved in agrochemical development within Europe have an understanding of English, but a translated version in the local language at the test site can avoid confusion and ensure successful study conduct in the field. Amendments to the study plan should be justified and approved by dated signature of the Study Director and maintained with the study plan. Deviations from the study plan should be described, explained, acknowledged and dated in a timely fashion by the Study Director and/or any Principal Investigators, and maintained with the study raw data. The study plan should contain, but not be limited to, the following information:

- identification of the study, the test item and the analytical reference item
  - (a) a descriptive title
  - (b) a statement which reveals the nature and purpose of the regulatory study
  - (c) identification of the test item by code or name [International Union of Pure and Applied Chemistry (IUPAC), Chemical Abstracts Service (CAS) number, biological parameters, etc.]
  - (d) the reference item to be used

- information concerning the sponsor and the test facility
  (a) name and address of the sponsor
  (b) name and address of any test facilities and test sites involved
  (c) name and address of the Study Director
  (d) name and address of any Principal Investigator, and the phase of the study delegated by the Study Director to the Principal Investigator
- dates
  (a) the date of approval of the study plan by dated signature of the Study Director
  (b) the proposed experimental starting and completion dates
- test methods
  reference to Organization for Economic Cooperation and Development (OECD) test guideline or other test guideline or method to be used
- issues (where applicable)
  (a) the justification for selection of the test system
  (b) characterization of the test system, such as the crop type, species, source of supply (if planting of seed is being used, e.g., seed treatment trials), number of trials, placement of trials, and other pertinent information
  (c) the method of application
  (d) the application rate and/or concentration, frequency, interval of applications
  (e) detailed information on the experimental design, including a description of the chronological procedure of the regulatory study, all methods, materials, and conditions, type and frequency of analysis, measurements, observations, and examinations to be performed, and statistical methods to be used (if any)
- records
  a list of records to be retained.

3.4 Product use pattern

Since residue kinetics are influenced by a variety of factors (e.g., the properties of the test item, morphology and physiology of the plant, agricultural conditions, soil properties, climatic conditions, crop cultivation methods, and application techniques), the study plan should allow for variation of these test conditions in order to keep experimental errors as low as possible. The study plan should ensure that the study is conducted in accordance with the procedures of Good Agricultural Practice (GAP) to ensure a healthy crop. Product labels must be followed regarding the handling, storage and application of the products so that the maximum rate is not exceeded and that the label use directions are followed in all test locations in order to generate consistent residue data. The GAP intensity may vary from country to country; hence, the Study Director and Principal Investigators must ensure that GAP is being followed where possible to the same standards in each country.

3.5 Test site requirements, evaluation and selection

A detailed description of the test system (crop) should be given in the study plan. Information on the growth stage and development of the crop (especially for the period

of the time of application) must be provided. Residue trials, which are conducted in open-field situations, should generally include data from four different sites in the same growing season. The sites should be a minimum of 15 km apart. The design of trials should take into account all the characteristics of a given crop and/or variety, which may have an influence on the uptake of the test item and upon the formation of residues. As far as possible, the common varieties of a crop should be used.

For applications made in the glasshouses or from post-harvest treatments, a single season is usually adequate since the geographical distribution of trial sites is immaterial. Specific conditions which affect cultivation (heated glasshouse, cold glasshouse, hydroponics) should be considered. These climatic conditions in protected crops usually lead to higher residues compared with the open-field situations.

Trials on cultivated plants performed in regions where these plants are the dominant crops should reflect the main types of agricultural practice. In general, for a comparable set of conditions, the four trials mentioned above should be repeated over a minimum of two growing seasons resulting in a total of eight trials per crop over the two seasons. The precise number of trials necessary for a new product is difficult to determine in advance of a preliminary evaluation of the trial results. Minimum data requirements only apply where comparability can be established between production areas, e.g., concerning climate, methods of production, etc. Assuming all other variables are comparable, a minimum of eight trials representative of the proposed growing area is required for major crops. For minor crops, normally four trials representative of the proposed growing area are sufficient. The number of studies per growing season can be reduced if the residue levels in plants/plant products can be shown to be lower than the limit of quantitation.

Where a significant part of the consumable crop is present during the application, half of the trials reported should include data on the residue level present over time (residue decline studies). The number of decline trials may be reduced if it can be shown that the edible part of the crop is not affected or present at the time of application of the test item and no movement of the active ingredient or its metabolite occurs.

Processing studies may also be required if the crop of interest is subsequently processed into commodities for human consumption (e.g., fruit juice, pomace, preserves, etc.).

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4 Best practices to conduct field studies

4.1 Evaluation and selection of field investigators and testing personnel

When the trial site is not on a special research facility, the evaluation and selection of field investigators (farmers) may be difficult. The best trial results are normally obtained from those trials conducted on farms where the farmers or technicians/agronomists involved with the normal application of pesticides on the farm are involved in the conduct of the trial, and are aware of the objectives so that they avoid treating the trial area with products likely to interfere with the analysis.

The agreement of the farmers or technicians/agronomists to conduct the trial, together with any payment for the use of the land or crop for the study, should be

established in a simple contract at the outset. The production of a working schedule outlining what is expected during the course of the trial, together with any restrictions or proposals for pesticides which can be applied to the trial area, should be agreed, and written instructions in their native language should be provided for guidance.

4.1.1 Training

The responsibility and acquisition of the trial site are normally left to the Principal Investigator for the field phase of the study. Therefore, a good knowledge of agricultural practices should be apparent from the training records of these staff. In some countries, such as the UK, specialized courses are available for staff to be trained in agricultural techniques and procedures. These staff should be trained in the use of application equipment and sampling equipment with appropriately designed SOPs to cover the field phase of studies. Specialist courses aimed at various application techniques can be followed and should be prominent in field Principal Investigator or Study Director training records. These records should also cover sampling techniques for various crop, water, soil, or clothing matrices.

4.2 Preparation of field testing study plan

As mentioned earlier, a written plan must exist prior to initiation of each regulatory study. The details of this study plan may be taken and used to form the details of a more specialized field study plan. The creation of these field study plans is common where a number of field investigators are involved in the conduct of the study. These field phase plans are in a form commonly used by the field staff and are often in the native language of the field staff. However, the study plan produced by the Study Director supersedes any field plan produced. The Principal Investigator and his QA must ensure that the field plan conforms with the original study plan and has signatures to verify this fact. The field study plan should be approved by dated signature of the Study Director. The field study plan should contain practical instructions concerning the conduct of the field study. Deviations from the field study plan should be described, explained, acknowledged, and dated in a timely fashion by the Study Director and/or any Principal Investigators and maintained with the study raw data.

4.3 Test item (previously termed test substance)

As a rule, residue trials are conducted only with the formulations or types of formulation proposed for registration or re-registration. Different formulations or mixtures may, however, be used in different countries. The details of the relevant formulation should always be presented in the study plan.

The name, the type and the batch number of the test item should be supplied with the formulation. A GLP (conducted to Good Laboratory Practice) Certificate of Analysis (C of A) detailing the above and also providing confirmation of the amount of active ingredient present in the particular batch of test item to be used in the study should be detailed. This description should include the date of receipt, the amount received,

storage conditions, place of storage, and expiry date. Data should also be available to indicate the stability of the test item formulation when mixed with water. The Study Director should ensure that appropriate analytical information is available and archived for each test item.

Experience has shown that when conducting multi-site residue studies in a number of countries a coordinated approach of centrally weighing and despatching the test item in pre-measured aliquots (together with copies of the relevant test item data sheets and dose weighing) provides an easily traceable and accurate accountability for the test item. The amount of test item required for field application is weighed from a central controlled point. This enables data to be generated centrally which verifies the quantity of the test item supplied to the field Principal Investigators to be used in the study. Disposal of any remaining test item can also be recorded more easily using this system. Field Principal Investigators are then required to record locally the data on their particular aliquot of test item for storage, person using the aliquot in the field, time of mixing, calculation procedures, and dilution steps involved for the application.

4.4 Trial layout

The size of the area used in the trial will vary from crop to crop. The size should be large enough to reflect routine use of the test item and allow collection of representative samples. As a rough guide, 25% of the crop should remain at the end of the sampling period. The experimental plot should be large enough to avoid contamination during hand or mechanical harvesting/sampling. This should also apply to control plots which should be situated in the immediate vicinity of the treated area so that cultivation and cropping can take place under identical conditions as far as possible. Sufficient distance between the test plots to avoid cross-contamination resulting from drift must be provided. In order to ensure that the trial plot layout is square (at 90°), a simple and easy method is to create a 3, 4, 5 right-angled triangle at the corners of the trial using a tape measure and canes as a marker. Mark 3 m along the base direction, 4 m along the vertical direction, and the resulting hypotenuse should be 5 m if the corners are at 90°. The lines to follow for the vertical and horizontal plot line when marking the exact plot dimension can be followed through these marker canes.

In the case of tall-growing plants and trees, provision should be made for one or more buffer rows or suitable protective shields, depending on the anticipated degree of drift.

When testing compounds with a high vapor pressure for use in glasshouses (fumigants, aerosols, smoke generators or fogging materials), care should be taken to obtain control samples from separate glasshouses. The use of Global Positioning Systems (GPSs) is now becoming more accurate and allows the corners of trials to be logged and recorded automatically so that trial sites are made retrievable without the need for triangulation to fixed points.

A description of the trial area, which includes a layout of the plots, the previous history of the trial area, a description of the plots, special local features, and the ownership of the land, should be recorded. Increasingly, digital photography is used to document site location, type of application used, crop growth stage, etc.

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Plots should be clearly labeled, and the trial/study identity should be marked in the vicinity of the trial. Plots should be clearly triangulated to fixed points so that at a later date independent third parties can identify separate plots on the basis of documentation.

Species and variety of the crop and the cultivation method employed may have a significant impact on the residue pattern. Therefore, the selection, sowing, planting, treatment, and care of the crops should be described in detail and should reflect the methods typical of the region in question.

4.5 Growing and maintenance of trial site crops

Prior to setting up the trial, site histories should be evaluated to ensure that chemicals applied previously do not influence the outcome of the planned analysis of the residues. Methods of cultivation should be identical for both treated and untreated plots.

The crop must be maintained in a healthy condition, free from disease and pests during the conduct of the study. To this end, application of crop protection products other than those being tested may be required. These compounds must not interfere with the determination of the residue. These products should be applied to both treated and control plots.

A record of any irrigation schemes (type of system used and amount of water) used during the conduct of the trial must be recorded. Often, fertilizers and maintenance chemicals may be mixed into and delivered with the irrigation water, and, hence, details must be recorded. In the case of long-term studies such as soil dissipation or accumulation studies, additional information about the water being used for irrigation (pH, major ion constituents, etc.) must be recorded.

These maintenance applications are often made by the farmer/cooperator, and these need to be documented. Often a contract can be drawn up with the grower, explaining carefully to him or his technical advisor what the objectives of the study are at the start so that no contamination occurs as a result of maintenance applications by the grower.

4.6 Calibration/servicing of application equipment

Calibration of application equipment must be performed for each study being undertaken. The use of good housekeeping techniques such as regular servicing and repair or renewal of equipment generally enables calibration to be performed with minimum inconvenience. Any equipment used (farm equipment, specialized field trials equipment, etc.) must be shown to be accurate and in good working order prior to its use in the application of the test item. Calibrating equipment prior to departing to the field provides a more controlled environment with all the necessary equipment available (flat surfaces, measuring cylinders, constant water supply, etc.) and is normally easier and more accurate than calibrating in the field, although calibration in the field is possible and may be necessary in some cases. In all cases, sufficient time should be allowed prior to application to perform the calibration successfully. Calibration should reflect the proposed application method.

The use of small plot trial equipment specifically designed for trials work usually has an advantage over commercial equipment when calibrations must be performed.

In the cases where liquid formulations are applied, calibration is normally performed by collecting the output volume over a given time period. Generally a minimum of three such measurements should be taken in order to estimate output consistency. Where output is collected from multiple nozzles or outlets, each nozzle or outlet should be evaluated in order to ensure uniformity of output across all the nozzles or outlets. If the deviation from the manufacturer's recommended value is not within ±5% (or the value specified in an appropriate SOP), the nozzle or outlet should be replaced. The use of a patternator allows the droplet distribution pattern of the nozzles or outlets to be measured accurately, and this check should be conducted annually. Having estimated the output of the equipment, the time required to treat a specific area with a known quantity of test item solution can be calculated.

The factors of walking or tractor speed, nozzle output, pump pressure, and width of treatment can be altered to provide the most accurate application method. As a preference, it is suggested that the output of the nozzles be set to the manufacturer's recommended output, and the speed of walking or driving be adjusted up or down to achieve the desired application rate.

Calibration of machinery for granular application is generally more difficult. Machines used in granular testing trials are often of a commercial scale requiring a larger quantity of test item to calibrate the equipment and to fill the reservoir of the machine. Normally, the output is governed by rotation of the equipment wheels, so the machine must be lifted from the ground in order to allow the wheels to rotate freely. A set of gears linked to the wheels generally allows the output to be increased or decreased as necessary.

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4.7 Test item application

A large range of equipment and types of application procedures are used in the agrochemical industry, and field trial techniques, where possible, should mimic these practices as closely and as accurately as possible.

The rate of product to be applied is the critical factor affecting all residue studies. The rate should be the highest recommended rate for that particular crop and should be applied at the limit of the GAP for the specific crop. The test item should ideally be pre-weighed in a laboratory prior to making the application. In most cases, this procedure results in easier accountability of test items, more accurate measurement of required doses, and more accurate application in the field since only the correct amount of water is required to be added by the field operator. This procedure also reduces the quantity of test item required and hence reduces the waste, which has to be disposed of. An additional spare sample, which is weighed at the same time in case of mishap with the original sample may be prepared.

Application rates in protocols can be expressed in various ways, and the field staff should be aware of these types of expressions and understand their meanings, e.g.,

- grams of active ingredient per unit area (g a.i. ha^{-1})
- grams of active ingredient per unit water volume (100 L) (g a.i. hL^{-1})
- grams of formulated product per unit area (g form ha^{-1})
- grams of formulated product per unit water volume (100 L) (g form hL^{-1}).

The list can be extended for each and every situation where plant protection products are used. There is an ongoing debate as to how to treat three-dimensional crops such as trees. The first approach is to estimate the number of trees in a given area, e.g., number per hectare. Having estimated the number of trees, the required water volume, e.g., 1000 L ha^{-1}, can be divided by the number of trees to ascertain the quantity of solution that each tree should receive. Knowing the calibrated output of the equipment, the required time to spray the required output per tree can be calculated. The disadvantage of this method is that each tree, whatever the size, receives the same amount of solution.

The second approach is to use a specified concentration of solution. This concentration is normally expressed as a hectolitre concentration and is the grams or milliliters of formulated product per 100 L of water. Here the trees are sprayed until run-off (the point at which the droplets coalesce and start to drip from the leaves). Once this point has been reached, the trees cannot be overdosed, since any additional solution will fall from the trees. This method, therefore, gives the advantages of (a) not overdosing, (b) tree size is irrelevant, and (c) no calculation of tree numbers is required.

The second approach is now becoming more widespread in its use but requires the product label to have the concentration specified as the hectolitre value for the farmers to use.

Whenever applying a test item, some important rules apply:

- check that the correct test item is being used and compare this with what is stated in the study plan. Often when coded products are being used, the number of the code may be very similar to others being tested, differing by only one or two letters or digits. The study plan may have been written by someone not responsible for sending the test item and hence may not, for instance, be fully aware of the full labeling on the container
- check that the dose that is pre-weighed has been calculated correctly
- check that the water volume to be used corresponds with that in the study plan
- check that the water volume to be used for mixing the test item is correct and has been calculated correctly and weighed or measured correctly
- it is good practice to have all the dose calculations checked by a second person prior to the application being made. The use of standard pre-printed forms or computer spreadsheets can often prevent unnecessary errors in calculation
- if it looks and feels wrong, it probably is! Remember that there is often only one chance to get it right, and failure could lead to a loss of 1 year of registration. If the trial is sprayed incorrectly, then the time and effort associated with the sampling and analysis of the samples are wasted.

Having received the pre-weighed test item, preparation for its use in the field must be made. Ideally, water to be used in the dilution of the test item should be from mains water or a recognized source. The use of water from standing pools, rivers, etc., could potentially lead to problems with interference from contaminants during analysis of the crop samples. Depending on the formulation under test, the test item can be mixed in a variety of ways. First, the required water volume must be accurately measured. Approximately half of this amount can be poured into a clean bucket or similar mixing container. The temperature of the water should be noted at this point

so that if there are any problems with the mixing and dispersion of the test item, this information is available.

Liquid formulations of test items are poured into the water and thoroughly mixed. The test item container should be rinsed thoroughly using the remaining half of the clean water to transfer any remaining test item to the mixing container. The complete amount of water and test item can then be mixed using a kitchen whisk or similar mixing tool. The time of mixing the test item for application should be recorded in the data. Where adjuvants or surfactants are being used in conjunction with the test item, these should be mixed first with the first half of the water prior to adding the test item.

Wettable powder formulations can be mixed using the procedure described above with the exception that some of the second half of the water should be used to 'cream' the test item into a paste type mixture which can then be poured into the first half of the water. Numerous rinsings will again be required to ensure that the entire test item is removed from its original container and, hence, thoroughly mixed with the second half of the water.

Dispersible granular formulations can be mixed using the procedure described above with the exception that all of the pre-measured water can be poured into the bucket or container. The test item granules are then poured into the water and allowed to dissolve for a few minutes before mixing. In some rare instances, the test item may be difficult to get into solution, and any problems should be recorded.

Experience has shown that in some instances mixing or creaming of the test item with a small amount of warm water can often aid the mixing procedure. Any difficulties in the mixing procedure and any steps taken to rectify the problem should be recorded in the data. Once mixed, the test item can be poured through a suitable filtering funnel into the relevant applicator, ready to apply to the test system. Application should be made in such a way as to mimic as closely as possible commercial practices. The use of practice runs (without spraying or spraying water only) in areas of the field close to the trial area can be a good way of helping to ensure that accurate application is performed. In order to measure what amount of test item has been applied to the trial area, either record the time taken to spray the trial area, knowing the output of the application equipment from the calibration, or measure the amount of test item remaining in the applicator, having known the initial volume (taking into account any test item remaining in the pipework of the application equipment or used to prime the application equipment before treatment). Safety equipment (e.g., face shield, gloves, spray suit, etc.) should be worn by applicators to ensure that they comply with Health and Safety Regulations.

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4.8 Sampling of crops

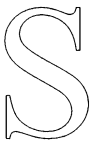
Guidelines for the methods of sample collection of crop samples are detailed in the Codex Alimentarius, but generally in most instances crop samples should be representative of the crop being grown. As a general rule, the quantity of sample required is a minimum of 12 units or >1 kg of field sample, e.g., potato tubers, cabbages, etc. Samples selected should not be damaged or suffer from severe defects, disease symptoms, or other abnormalities.

The best way to collect accurate information on the fate of test items, and hence their residues, would be to analyze the total yield of a plot. This is, of course, not feasible. Therefore, the only manageable solution is to collect representative samples from within the treated and untreated plots.

4.8.1 Sample collection in the field

When choosing sampling locations and methods, take into account the factors which influence the crop, e.g., the plant morphology, differences in plant growth, the way the plot was treated, etc., all of which have an influence on the distribution of residues both on individual plants and in the trial plot as a whole. The actual material required to produce a field sample can be selected, as a rule, by one of the following methods:

1. Random
   Samples are collected with a free choice of the sampling places within the respective plot. Care must be taken to avoid the plot boundaries and margins.
2. Systematically
   Samples are collected along a defined pattern such as an 'X' or an 'S' or in the case of narrow plots starting from the edge of the plot and working across the plot:

'X' Pattern	'S' pattern	From edge of plot
X X X X X X X X X X X X		Outward route —X—X—X—X— —X—X—X—X— —X—X—X—X—X— —X—X—X—X—X— Return route

Samples should be carefully collected to avoid contamination between treated and untreated samples. The following rules should be followed to reduce the risk of cross-contamination:

- always collect samples from the untreated control treatment before the treated samples are collected
- wear disposable gloves, coveralls, etc., as necessary, depending on the crop type
- avoid walking through the treated plot unnecessarily and, if doing so, ensure that disposable clothing is changed before entering the untreated control plot
- if possible, have two staff members collecting the samples – one sampling the untreated plot and the other the treated plot
- if possible, use separate designated sampling equipment for untreated control samples and treated samples. Ensure that these are clean prior to sampling, and if working in different plots with the same equipment, ensure that the equipment is cleaned after use between samplings

- if using larger equipment for sampling, e.g., a small plot combine where having two machines is impractical, ensure that point 1 is followed, and clean the machine thoroughly prior to collecting or processing the next sample. Passing untreated 'discard' material through the machine between treatments decreases the likelihood of contamination.

4.8.2 Sample preparation in the field

Excess soil from samples such as a root crop should be removed with light brushing but never washed prior to placing the samples into collection bags. In the case of mature root crops, e.g., sugar beet, the roots and leaves should be processed separately, and the weights of leaves and roots should be determined separately in the field.

Leaf/plant material, which is inedible and does not normally constitute the commodity, can be removed in the field.

Where samples are large, e.g., cabbage, melons, etc., the unit (cabbage) may be cut into quarters. Select the opposite quarters of the unit sample, which are then used to make the final composite sample, thus reducing the sample volume by half for easier handling, freezing, and shipping of the samples.

For stone fruit, e.g., olive cherries, where the mature fruit is analyzed, the stone should be removed, and the weight of pulp and stone should be recorded. The residue is calculated on the basis of whole fruit. This step can be done either in the field prior to the fruit being frozen, which makes the procedure easier, or in the analytical laboratory. In either case, care needs to be taken to avoid cross-contamination.

Large-stem samples (e.g., maize) should be collected (12 units) and then divided into three parts, all of approximately the same size. The upper parts from stems 1–4, central parts of 5–8, and tops of 9–12 can be combined to form the one sample.

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4.8.3 Sample intervals

Where samples are being collected to show or investigate the change in residue with time, a minimum of five time points should be used where samples are collected. This should cover the time from just prior to the last application to the final PHI. An example of the sampling regime would be to collect a sample prior to the final application and again once the test item has dried onto the crop following the final application. Subsequent samplings (a minimum of three) would be targeted to cover the possible harvesting period. If the crop maturity/harvest extends over a wide range, additional sampling points beyond the normal or expected PHI may be used. If the mature fruit or main harvestable commodity is not present at the sampling point, suitable samples, e.g., whole plant, pods, etc., should be collected.

Where trials are conducted to determine the specific harvest period, samples can be collected at the targeted PHI only.

Experience has shown that confirmatory data relating to the amount of test item applied can be gained by the collection of samples immediately following the final application.

Where trials are required to investigate the possible residue of test item in processed samples, e.g., juice, purée, etc., a larger quantity of the field sample must be collected. To collect this larger sample size, the size of the plots must often be increased at the

outset of the trial. The magnitude of the sample is dependent on the processing procedure required to generate the final processed commodity, but would generally range from a minimum of 2 kg (cucumber, artichokes) to 25–30 kg for wine grapes. Samples for the processing studies should be collected at the targeted PHI in order to achieve a worst-case situation.

4.8.4 Packaging/labeling and transport of field samples

When selecting materials for packaging and storing samples, ensure that they will not interfere with the analysis, e.g., through migration or interfering substances from the packing material into the samples or adsorption of residues to the packing material. Paper bags with an inner lining of aluminum foil have proved to be especially suitable. Other materials can be used as substitutes, e.g., sturdy polyethylene bags or, for less bulky samples (rapeseed, cereal grains, etc.), polyethylene boxes (lined with aluminum foil, if required). After collecting samples in the field, the samples should generally be placed in suitable bags to hold the weight of the sample without breaking the seal or piercing the bag. As good practice, the sample should be double bagged in order to prevent contamination. In some instances where the residue may be volatile or may adhere to plastic, the sample may be collected into paper or Teflon-coated bags. The bagged samples can then be stored collectively in cardboard boxes, according to treatments (untreated and treated separate). These boxes should be sturdy, be of a suitable size to hold the relevant samples, and be suitable for stacking in frozen storage or for shipping via either road or air freight.

In the case of processing study samples, the samples to be processed should be collected fresh and remain fresh (never frozen) in the type of bag, container, etc., normally used for transportation of these crops from the field in commercial practice, e.g., paper bags, net bags, boxes, and trays. This should prevent deterioration of the samples and mimic commercial practices.

Suitable labels placed strategically on the bag should uniquely identify each sample. Placing a label between the first bag and the outer bag allows the label to be clearly observed at all times. This also avoids placing a label in direct contact with the sample where interference may be transferred to the sample from the label, or the information may be rubbed off during contact with the sample. A second label tied to the first bag ensures that the label cannot be lost following sealing of the first bag within a second bag. The use of color-coded labels, e.g., blue for untreated and red for treated samples, enables the distinction to be made easily between the two types of sample when being handled and upon receipt by the analytical laboratory. The labeling system employed varies hugely from one organization to another. A system that enables the field operator to see at a glance what type of sample he or she has to collect and at what sampling point this should be collected is preferred. An example of the annotation for a label could be as follows:

396 007/1/S1/101/tubers where 396 007 = study number
1 = trial number
S1 = sampling time point
101 = plot number (treatment)
tuber = crop part to be collected

Often where direct data capture systems are employed in the analytical laboratory, an additional bar code or sequential labelling system may be incorporated and could be added to this system to ensure complete union with the analytical laboratory receiving the samples.

Samples collected from the field should ideally be placed in boxes in order to prevent damage to the crop sample and to aid storage, although this often depends on the freezing facilities of the organization concerned. Where samples are boxed, untreated samples should not be mixed with treated samples. When freezing, samples should be separated by space or by using separate freezers for treated and untreated samples.

4.9 Sample shipping and transportation

During transportation, make sure that the samples are protected against any alterations in the residue situation (e.g., by spoilage, damage, or contamination or by changes in the moisture content of the matrix and degradation of the residue). Ensure also that the sample labels remain intact. Any gaps between the individual samples in the container should be stuffed with extra filling material (e.g., paper, foil).

If samples are transported frozen, the packaging containers should be made of a hard and impact-resistant material such as polystyrene. Cardboard cartons with insulating material can also be used.

To ensure that the samples are still frozen on reaching their destination, adequate insulation and a sufficient supply of dry ice are absolutely necessary. A minimum of 1 kg of dry ice per kilogram of sample material is normally needed, but the amount must be increased if the containers are poorly insulated.

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Frozen samples must not be allowed to thaw during transportation. Care should always be taken when handling dry ice (−70 °C). Gloves should be worn, and the workplace must be adequately ventilated since there is a danger of suffocation! Do not transport packages containing dry ice in passenger cars; the containers need to be sealed away from passengers.

Packaging must comply with existing transport regulations. If necessary, labels must be attached to the outside of the dispatch container with warnings such as the following:

Perishable Goods. Deliver Immediately on Arrival
Contents for Scientific Purposes Only
Dry Ice. Danger of Suffocation

4.9.1 Transportation

Good rules to adopt when transporting samples are as follows:

- notify the recipient of dispatch
- ensure that the required documents (Sample Chain of Custody) are enclosed
- ensure that the samples reach their destination as rapidly as possible
- ensure that the samples can be clearly assigned on arrival.

Do not dispatch samples less than 2 days before a weekend or a public holiday to avoid unexpected delays. Should the samples arrive outside working hours, provision must be made for suitable intermediate storage. Often this is left to the responsibility of security staff, who may not be aware of the importance of the frozen samples.

The type of carrier, the route, and the time needed for transportation should be known to the sender and also be communicated to the recipient. In the case of air-freight, for instance, the recipient should be given details beforehand about:

- the airport destination
- the date of arrival
- the airwaybill number
- the airline
- the flight number.

All quarantine and customs regulations must be observed if samples are dispatched across the national frontier. A 'phytosanitary certificate' may be needed and should be secured in good time before the proposed date of dispatch. The EEC generally allows free movement of goods within the EU, although moving from non-EU to EU countries does result in more stringent paperwork.

Transport of samples to the analytical laboratory presents the staff of organizations conducting field trials with the most difficult problem and is the area where many studies have failed as a result of samples being lost, defrosted, or shipped to the wrong place. The number of experiences and ill fortune that have befallen many Field Trial Managers are too many to mention.

There are generally two ways to ship samples to the analytical laboratory within Europe. The first method involves shipping frozen samples in the presence of dry ice. These samples are generally shipped by airfreight to the analytical laboratory. The second method is to ship by frozen transport via the road. The two methods of shipment both have some advantages and disadvantages. Table 1 highlights the two scenarios.

4.10 Sample storage

The samples should be analyzed as soon as possible after they have been taken, before physical or chemical changes can occur. For this reason, any intermediate storage of nonfrozen field samples should be avoided as far as possible, and dispatch of samples to the receiving laboratory should, as a general rule, take place immediately after sampling.

Light can result in degradation of the residues; hence, the samples should be protected from any avoidable exposure, e.g., by the use of suitable packaging material.

If brief intermediate storage of nonfrozen samples is necessary, a cool and dark place should be chosen for the purpose. In the case of frozen samples, make sure that there is no interruption in the refrigerated conditions up to the time of analysis or processing, and that the package is well sealed to prevent the samples from drying out in the event of prolonged storage.

Table 1 Advantages and disadvantages between two methods of shipping samples to the analytical laboratory within Europe

Air freight		Ground transport (temperature controlled)	
Advantages	Disadvantages	Advantages	Disadvantages
Rapid delivery of samples			Slow delivery of samples
Cost relatively low since sample size is limited			Cost relatively high owing to time spent on the road
Lack of control/accountability of samples		Improved control/accountability of samples, especially if own transport is utilized	
	Difficult to obtain dry ice, especially in southern Europe	No dry ice required	
	Possible delay at airport customs as they check for dangerous goods, etc.	Regulations within the EEC now allow for movement of goods within EEC	Note that Switzerland is not part of the EEC and hence should be avoided for a passage through to another destination, since long delays due to paperwork formalities will be encountered
	Sample size is restricted by the volume/weight of dry ice which can be carried on one airplane	No restriction except by the size of the freezer transport used	
	Agent required to process samples through customs at both the departure and arrival airports	No agents required although paperwork stating goods of no commercial value are being transported is needed. Also paperwork is required by the haulier	
	Secondary transport to be arranged from airport to analytical laboratory	Single transport source is responsible from field to analytical laboratory	

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The samples are identified in the receiving laboratory on arrival on the basis of documentation provided in the shipment and the information supplied in the study plan and chain of custody. Acknowledgement to the field investigator can then be given.

4.11 Record keeping

(1) Field notebook

As a general rule, data recorded in the field are generally documented on standard forms. The use of pre-formatted field forms into a 'field notebook' allows data to be recorded and kept in a uniform manner for ease of reporting at a later stage. These notebooks should have numbered pages and be bound in order to prevent removal or addition of pages. Since all reports are normally presented in English, the field notebook is normally also presented in English. The field notebooks for some organizations exist in dual languages, so that the field staff in remote stations have the ability to conduct and record the study in their own language. Electronic versions of field notebooks are also becoming more common. The use of such systems provides an easy way of communicating with large numbers of organizations who could potentially conduct the field trials work. However, these electronic systems need to be validated and shown 'fit for use' with the appropriate protection, accountability, and traceably in place prior to use.

(2) Crop, field application and sampling information

Details of the test system (crop) and background data surrounding the study should be documented. The data recorded should include the following:

- trial location (including post code)
- soil type
- soil moisture (e.g., dry, damp, moist).

For certain types of study, e.g., soil dissipation, additional factors may need to be recorded, e.g., soil biomass, specific soil moisture content, soil conductivity, and pH.

- crop
- crop variety
- crop density (seed rate, planting rate)
- planting date
- flowering date during the conduct of the study (where applicable)
- harvest date
- previous crop history and maintenance chemicals used
- growth stage at each application and sampling [preferably using the Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenant und Chemische Industrie (BBCH) scale]
- percentage ground cover of crop (for two-dimensional crops)
- crop moisture during application and sampling (e.g., dry, damp, moist)
- spray equipment used
- nozzle type/number used

- output volume/rate
- walking/application speed.

(3) Weather data

Critical weather data are recorded during the application procedure. The data recorded should cover the following, but additional data may also be recorded as necessary. Within Europe, the scales in parentheses are normally applicable:

- air temperature (°C)
- soil temperature (°C at 10-cm depth)
- wind speed (m s^{-1}) recorded at the height of the application equipment preferably at the time of application
- wind direction in terms of compass direction (N, S, E and W)
- wind direction in relation to the test system
- relative humidity (%).

Ideally the time of application should be available in the data, and the treatment should be made under good conditions for application.

Other data such as light intensity, percentage cloud cover, and soil moisture may also be recorded. The use of meteorological equipment, which can measure critical climatic information such as wind speed (current, maximum and average), humidity, air temperature, and dew-forming point, is preferable in the field. Historic weather data in the form of 10-year averages must be reported and are required for comparison of the trial specific data with the 'normal' weather data.

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A system of record keeping must be used which eliminates any risk of confusing the samples. As a rule, samples should be recorded immediately after their arrival and, especially in the case of nonfrozen field samples, prior to preparation for analysis. A record should also be kept of the condition of the samples upon arrival at the receiving laboratory and of any packaging or labeling defects.

Since there are a large number of companies, both contract organizations and agrochemical manufacturer organizations within Europe performing these operations, the working practices vary over the range of companies, but the basic data being recorded should be the same.

(4) Field data report

A final report should be prepared for each regulatory study. In the case of short-term studies, a standardized final report accompanied by a study-specific extension may be prepared. Reports of Principal Investigators or scientists involved in the regulatory study should be signed and dated by them. They should contain a Principal Investigator statement and QA statement if the Principal Investigator is not linked to the Study Director organization.

The final report should be signed and dated by the Study Director to indicate acceptance of responsibility for the validity of the data. The extent of compliance with the principles of GLP should be indicated.

Corrections and additions to a final report should be in the form of amendments. Amendments should clearly specify the reason for the corrections or additions and should be signed and dated by the Study Director.

Reformatting of the final report to comply with the submission requirements of a national registration or regulatory authority does not constitute a correction, addition, or amendment to the final report.

The final report should include, but not be limited to, the following information:

1. Identification of the regulatory study, the test item, and the reference item
   (a) a descriptive title
   (b) identification of the test item by code or name (IUPAC, CAS number, biological parameters, etc.)
   (c) identification of the reference item by name
   (d) characterisation of the test item including purity, stability, and homogeneity.
2. Information concerning the sponsor and the test facility
   (a) name and address of the sponsor
   (b) name and address of any test facilities and test sites involved
   (c) name and address of the Study Director
   (d) name and address of any Principal Investigators and the phase of the study delegated, if applicable
   (e) name and address of scientists having contributed reports to the final report.
3. Dates
   Experimental starting and completion dates.
4. Statement
   A quality assurance program statement listing the types of inspections made and their dates, including the phases inspected, and the dates when any inspection results were reported to management, to the Study Director, and to any Principal Investigators, if applicable. This statement would also serve to confirm that the final report reflects the raw data.
5. Description of materials and test methods
   (a) description of methods and materials used
   (b) reference to OECD test guidelines or other test guidelines or methods.
6. Results
   (a) a summary of results
   (b) all information and data required in the study plan
   (c) a presentation of the results, including calculations and determinations of statistical significance
   (d) an evaluation and discussion of the results and, where appropriate, conclusions.
7. Storage
   The location where the study plan, samples of test and reference items, specimens, raw data, and the final report are to be stored.

5 Good Laboratory Practice

All UK facilities conducting regulatory studies must be members of the UK Good Laboratory Practice compliance program and all regulatory studies must be conducted

to the principles of GLP. The conduct of multi-site studies with one protocol covering the whole study is now the norm in Europe. The Study Director may be a person from an analytical or from a field background, but the appointment of well qualified Principal Investigators for either phase of the work reduces the likelihood of problems in either phase.

All phases of a multi-site regulatory study should be carried out in facilities that are members of the UK or a relevant national GLP compliance program. Pre-study test site inspections may be conducted if considered necessary. If an organization is considering using a particular test site, a copy of the test facility's current GLP certificate should be obtained and included in the QA multi-site file.

If the test facility claims to be GLP-compliant but is located in a country where there is no authorised body responsible for GLP monitoring, i.e., a national GLP compliance program, the Study Director needs to be assured that the facility (including the archive, if used) does operate in compliance with GLP principles. This can be achieved by conducting a pre-study QA test site inspection or by a review of documentary evidence, e.g., notice of adverse findings and subsequent responses, or Establishment Inspection reports. The Study Director should ideally discuss the above with QA personnel and send copies of any documentary evidence used to the Quality Assurance Unit (QAU) for inclusion in the QA multi-site study file.

In some instances, phases of a multi-site study may have to be conducted in a noncompliant facility, e.g., if there are no GLP-compliant facilities that can conduct the work. In the UK there are two options for dealing with this.

1. Extending the Test Facility (this may be a sponsor or contractor depending on the location of the Study Director) GLP system to cover the work. If the extension to the GLP system is required, the Good Laboratory Practice Monitoring Authority (GLPMA) should be notified by QA personnel, in writing, of the test site to be used, the regulatory studies concerned, and the type of work to be undertaken. The rationale for the selection of the test site should be documented, and the Test Facility of the Study Director must put QA and management mechanisms in place to ensure that the work is conducted in accordance with GLP principles. The work may be subject to inspection by the GLPMA, and additional cost implications may need to be considered.
2. Include a 'disclaimer' or deviation in the Study Director GLP compliance statement. The disclaimer route can be used for a phase of a multi-site study but should generally not be used if the work in question is a critical phase, e.g., application. The remainder of the study must be conducted in accordance with the principles of GLP. This route may not be acceptable to overseas regulators and should be avoided if possible.

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5.1 Field QA audits and study involvement

The Study Director/Principal Investigator should ideally discuss all multi-site studies with QA personnel prior to study initiation (or prior to the issue of the protocol amendment if work is not detailed in the protocol). When acting as Study Director, a copy of the current GLP certificates should be requested from sub-contracted facilities and should be retained in QA.

The QA monitoring of a multi-site study needs to be carefully planned to ensure overall compliance of the study. Responsibilities for this monitoring should be clearly defined in the protocol/amendment to prevent problems. Test Facility QA personnel may also make contact with the test site QAU to ensure adequate QA coverage. The QAU at the Principal Investigator site should send details of inspection and report audit findings to the Study Director and to the Study Director's management. This can be achieved by sending a copy of the actual QA report or a letter detailing a summary of the findings. QA personnel will normally send the actual inspection report and a summary of report audit findings. The Study Director and the Study Director's management should provide documentary evidence that they have reviewed QAU reports/letters from the test site. These reports/letters should then be sent to the QAU for inclusion in the relevant QA study file.

Test Facility QA personnel and test site QA personnel should sign and date QA statements detailing any monitoring that they carried out.

Inspections should be carried out as study-specific inspections for critical phases of the study or as a batch of process inspections relating to critical tasks which are performed regularly and although may not be inspected for a specific study are inspected on a regular basis (e.g., once per month). The coordination of inspections is perhaps more difficult to predict than actually conducting the critical event. The use of local QA staff close to the sites where the field work is being conducted helps to reduce the travel time to the field sites and also the down time if the critical event cannot be made at the specified time. The use of computer planning tools to schedule fieldwork is very helpful, not only to the field staff but also to QA.

The conduct of field GLP audits/inspections by QA staff can be one of the most difficult tasks to arrange and execute for multi-site field studies. Since the critical phases of such studies are generally dependent on the weather, predicting the exact date and time on which a particular event will occur can be very difficult. If QA staff are based remote from the field staff and the site, the logistics of sending QA staff at short notice to inspect a study can be a logistical nightmare. Therefore, having QA staff based near to the field operations in order to carry out such inspections is advantageous. The use of suitably trained consultant QA staff based locally to the trial may provide additional support at short notice. The use of Principal Investigators from a separate company, which has its own locally based QA staff, can also provide a useful alternative to sending QA at short notice. An alternative approach employed by some companies is to send QA staff to remote field stations on a pre-defined schedule throughout the course of a season. They then spend a significant quantity of time with the field staff and conduct both critical phase inspections (inspections relating specifically to studies, e.g., application) and process inspections (nonstudy specific inspections, e.g., data recording) so that the GLP integrity of the program of studies conducted by that field station staff can be assured.

6 Archiving

The length of data storage must be noted in the Study Plan (as a rule a minimum of 10 years for most of the countries within the EU). This, however, can differ between different countries within the EU. Where multi-site studies are conducted, the location of study raw data for each site must be specified. One preference is to have all original

raw data archived at the site of the Study Director. In this instance, retention of certified copies of the raw data at the various sites may be beneficial in order to remove the necessity to send original data back to the field sites when they are undergoing inspection by the national monitoring authority.

1. The following data should be retained in the archives for the period specified by the appropriate regulatory authorities:
   (a) the Study Plan, raw data field notebook, samples of test and reference items, specimens, and the final report of each regulatory study
   (b) records of all inspections performed by the quality assurance program, and also master schedules
   (c) records of qualifications, training, experience, and job descriptions of personnel (normally archived when staff leave the employment of the organization)
   (d) records and reports of the maintenance and calibration of apparatus
   (e) validation documentation for computerized systems
   (f) the historical file of all standard operating procedures
   (g) environmental monitoring records.
2. In the absence of a required retention period, the final disposition of any study materials should be documented. When samples of test and reference items and specimens are disposed of before the expiry of the required retention period for any reason, this should be justified and documented. Samples of test and reference items and specimens should be retained only as long as the quality of the preparation permits evaluation.
3. Material retained in the archives should be indexed so as to facilitate orderly storage and retrieval.
4. Only personnel authorized by management should have access to the archives. Movement of material in and out of the archives should be properly recorded.
5. If a test facility or an archive contracting facility goes out of business and has no legal successor, the archive should be transferred to the archives of the sponsor of the regulatory study.

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7 Conclusion

To conclude this section of best practices, two important pieces of advice in conducting field trials are:

- 'never put off until tomorrow what you can do today'. There are many occasions when field operation is delayed for 1 day. The weather conditions for the next day might not be favorable for the operation, and the designed protocol is deviated from, and this may affect the overall objective of the study
- remember when recording data that however trivial the data might appear, they are better recorded than not recorded and lost forever. These missing data may impact the overall acceptance of the study.

Further reading

Codex Guideline EMRL(1)-1997, 'List of Codex Extraneous Maximum Residue Limits in Food', Codex Alimentarius Commission, Washington, DC (1997).

Codex Guideline MRL(1)-1999, 'List of Codex Maximum Residue Limits for Pesticides Residues in Food', Codex Alimentarius Commission, Washington, DC (1999).

Commission of the European Communities, Directorate-General for Agriculture, 'Guidelines for the Generation of Data Concerning Residues as Provided in Annex II, Part A, Section 6 and Annex II, Part A, Section 8 of Directive Concerning the Placement of Plant Protection Products on the Market 1607/VI/97 Rev. 2 10/6/199, Commission of the European Communities, Brussels (1999).

Statutory Instrument 1999 No. 3106, 'The Good Laboratory Practice Regulations 1999', Stationery Office, London (1999).

Conducting crop residue field trials in Mexico and Latin America

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1 Introduction

Tolerances are required for all agricultural chemicals used on crops which become part of the diet consumed in the USA. If these crops are grown in the USA, and the sponsoring company decides there is a sufficient market for a US registration, they are included on the US label for this chemical and appropriate residue testing is conducted within the USA to achieve these tolerances. When, however, crops which become part of the US diet are grown completely or in large part outside the USA, residue testing must be done at the site of origin of the food commodity. Since roughly one-third of US imported food commodities originates in Latin America, agricultural chemical producers have found it increasingly important to develop the capability to conduct field residue testing, under US Environmental Protection Agency (EPA) guidelines, in Latin America.

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Agricultural chemical companies who operate internationally are finding that time and money can be saved by adopting a worldwide registrations strategy that allows for the use of residue tests conducted in one country to help satisfy the requirements of another. For example, since certain vegetables such as tomatoes, peppers and broccoli are grown in the USA and also imported in large quantities from Mexico, the opportunity exists to run a certain number of field residue tests in Mexico, in regions such as Sonora and Sinaloa states, which are climatically similar to southwestern US regions. These field residue trials could then, if both US and Mexican regulations have been followed, serve as part of a registration package for the chemical involved in both the USA and Mexico. Harmonization of the labels in the two countries is important in these instances to ensure that the same rate of product is being applied in all trials. Today, separate registration petitions would most likely occur in both countries, but in the future, these will occur as a joint US/Mexico North American Free Trade Agreement (NAFTA) registration, much as we now see joint US/Canada NAFTA registrations.

Where a crop which constitutes a part of the US diet is not grown in sufficient quantity in the USA to warrant the cost to obtain a US registration, an import tolerance is required. Crops that are grown extensively in Latin America, such as coffee, banana,

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and pineapple, are grown in relatively small quantities in the USA (Hawaii and Puerto Rico). If an agricultural chemical targeted for use on these crops is marketed outside the USA, a full tolerance, which allows for importation of crops treated with the chemical in question, will no longer be granted by the EPA if field residue tests are not conducted in the major crop-producing countries where the chemicals will be used.

Field residue studies in Latin America, whether they are run to satisfy requirements for an import tolerance or as part of a US tolerance program, must be based on EPA regulations and guidelines and conducted under Good Laboratory Practice (GLP) and Good Field Practice regulations. If a study deviates from GLPs, a statement must be included in the study stating any deviations and the effect on the study. Any deviations should be noted in the report. These requirements are described in Code of Federal Regulations (CFR) 40 CFR 160.1(a) and 160.3(4) and are available on the EPA web site (**http://ww.epa.gov**). While there are laboratories in Latin America which are capable of running chemical analyses under GLP regulations, normal practice for agricultural chemical producers is to have the treated commodity samples shipped to laboratories in the USA or Europe for analysis.

In the author's experience, field residue trials can be conducted in Latin America under complete compliance with all EPA guidelines and recommendations. More time does need to be spent in planning and preparation to ensure that the personnel involved in the testing have been trained in GLP and that this training, as well as the compliance of equipment, storage and archival sites, standard operating procedures (SOPs), and quality assurance (QA) audits, are properly documented.

2 Regulatory requirements

A very clear and well-written guidance document for import tolerances was published in June of 2000 in the *Federal Register* by the Office of Pesticide Products of the EPA. The document is available on the EPA web site through their *search* function under 'Import Tolerance Guidelines'. The complete web address is **http://www.epa.gov/fedrgstr/EPA-PEST/2000/June/Day-01/p13708.htm**, and the reference for the *Federal Register* is June 1, 2000 (Volume 65, Number 106, pages 35069–35090.

The term 'import tolerance' is used to refer to a tolerance that exists where there is no accompanying US registration. As the Import Tolerance Guidelines state, 'There is no statutory or regulatory distinction between an "import tolerance" and any other tolerance issued by EPA. The same food safety standards apply to tolerances proposed for both domestically produced and imported food; as a result, domestic and foreign growers are treated equally. Generally, tolerances are set for raw agricultural commodities and also apply to processed foods derived from the commodities.'

A large part of the US diet is made up of crops which originate outside the USA. Currently, a US tolerance achieved through the submission of data obtained from residue trials run exclusively within the USA permits the importation of commodities grown in Latin America or other countries. Within the past 5 years, the EPA has initiated programs to ensure that residue testing to achieve a US tolerance better reflects the climatic and cultural conditions under which the commodity is grown.

In cases such as tomatoes, peppers, broccoli and strawberries, to name a few, a high percentage of the quantity consumed in the USA originates in Mexico, particularly during the winter months. The Import Tolerance Guidelines (see above) explains the need for foreign residue data for both import tolerances and US tolerances for food crops with a 'significant import component'. The Guidelines state that the decision by the EPA as to whether or not foreign residue data will be required is based on the following types of screening information:

- what international tolerances or maximum residue limits (MRLs) exist?
- which countries export the commodity to the USA?
- major seasonal variations in imports of the commodity
- percentage of US consumption which is imported
- percentage of crop treated in the exporting countries
- significance of the food in the US diet
- effect of processing on the residues
- available information on levels of residues found in samples of imported food [based on Food and Drug Administration (FDA), US Department of Agriculture (USDA), or other monitoring data].

Generally, for a US tolerance, there will be a requirement for additional residue information (i.e., foreign residue data) only where:

- imported commodities comprise a high percentage of US consumption
- domestic residue data are not likely to be representative of growing conditions in other countries, or
- US consumers would likely be exposed to significant residues in imported foods.

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In the past, EPA has not specifically considered the contribution of residues in or on imported food when establishing (or reassessing) tolerances for US registrations.

The Import Tolerance Guidelines provide two examples of cases where foreign residue data may be considered for a US registration:

1. Cranberries: *Not likely* to require foreign residue data. 'Cranberries account for an extremely low percentage of the US diet. In this case, EPA would probably not require submission of foreign residue data because dietary exposure to residues in imported cranberries is very low and EPA determines that US field trials would be representative of growing conditions in Canada.'
2. Bananas: *Likely* to require foreign residue data. 'The vast majority of bananas consumed in the USA are imported. Bananas are imported from Central and South America, and cultural practices for bananas grown in the USA differ from those in Latin America. Existing residue data consist of five US field trials in Hawaii and Puerto Rico. Bananas represent a relatively high percentage of the US diet, especially for children. To assess the safety of the tolerance, EPA would likely require submission of additional residue data based on the pesticide's use in major banana exporting countries for the following reasons: most of what is consumed in the USA is imported and EPA has no data on such foreign uses; cultural practices in other countries appear to differ from those in the USA; and bananas represent a relatively high percentage of the diet of a potentially sensitive sub-population (children).'

If a US tolerance is desired for an agricultural chemical on a crop which is imported in significant quantities from Mexico (e.g., tomatoes, peppers, strawberries, broccoli, etc.), a development plan should be constructed which includes residue testing in those regions in Mexico which are deemed by the EPA to be similar to southern US regions in climate and crop cultural practices. This will not only prevent duplication of effort for the US registration, but carefully designed tests could then also be used to satisfy Mexican registration requirements.

In order to improve cooperation and sharing of data reviews for agricultural chemicals in the NAFTA countries, a North American Pesticide Initiative was created. This initiative was designed to assist in harmonizing data requirements and policies among the three countries. 'Zone maps' that will permit the use of data from similar growing regions in Mexico, Canada and the USA have been developed. The EPA has been actively working with the regulatory agencies in Canada and Mexico to establish a single set of residue guidelines for NAFTA, which will be based on the current US guidelines and best practices.

3 Planning a field residue trial in Latin America

3.1 Number and locations of trials

The required number and locations of trials must first be determined, based on the quantity and source of imported commodities. Information on agricultural imports may be obtained from the USDA, the US Department of Commerce, and various private sources. The web site for the Foreign Agricultural Trade of the US (FATUS) lists of imported and exported commodities is **http://usda.mannlib.cornell.edu/reports/erssor/trade/fau-bb/data**. In order to determine percentage of total domestic consumption, one also needs *Agricultural Statistics*, a yearly publication from the USDA. This is available through the National Agricultural Statistics Service of the USDA on their web site: **http://www.usda.gov/nass/**. The guidelines state that trials must be conducted in any country from which a quantity of 5% or greater of the domestic supply is imported (average of the five most recent years' available data). The number of trials required is dependent on the quantity of imports and the importance in the US diet.

Bananas are chosen for the example below, which is taken from the Import Tolerance Guidelines.

Approximately 99.8% of all bananas available in the USA are imported. The highest consumption level for any population sub-group is 0.96% of the diet for infants. Based on information given in the Import Tolerance Guidelines, a minimum of 12 trials would be required.

To ensure that all countries that account for greater than 5% of the amount imported are represented, and that the countries with the most production are most heavily represented, the 12-trial minimum (and 24 treated samples analyzed) could be distributed among exporting countries as listed below. Both bagged and unbagged samples need to be analyzed for bananas. The option is provided of analyzing one bagged sample and one unbagged sample from each site.

The list below indicates the countries and amounts of bananas imported into the USA (thousands of pounds, 1991–95 average):

Ecuador	2 076 329
Costa Rica	1 994 840
Colombia	1 312 890
Honduras	1 032 646
Guatemala	866 371
Mexico	559 385
Panama	191 409
Venezuela	11 416
Other countries	81 366

Since the USA imports less than 5% of its bananas from Panama or Venezuela, residue trials are not required in these countries. The 12 required residue trials would then be apportioned among the six largest exporters to the USA, listed below:

Ecuador	3 trials
Costa Rica	3 trials
Colombia	2 trials
Honduras	2 trials
Guatemala	1 trial
Mexico	1 trial

These trials should be placed within each country in the areas with the largest production. There are circumstances, however, under which countries can be excluded, even if their US crop imports are over 5%. For instance, if the petitioner does not market or does not intend to market the subject pesticide in one of the top two or three countries that export the crop to the USA, then the total percentage imported should not include the countries in which the pesticide is not marketed or intended to be marketed. Also, if natural disasters or political problems make it difficult to include a specific country, one may request that the EPA accept additional trials from neighboring countries which can be shown to have similar climatic conditions and cultural practices.

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3.2 Personnel requirements

Requirements for personnel involved with any field residue study, within or outside the USA, are documented in the GLP regulations (40 CFR 160.29). Personnel involved with the conduct of the study must be qualified to carry out the task they are assigned. All personnel contributing to the conduct of the study must be listed in the final report, and training records, job descriptions, and curriculum vitae for all study personnel must be available in the archives of the research unit responsible for the conduct of the study. Frequently, in many parts of Latin America, the Principal Investigator works with a crew of experienced farm laborers who operate the spray equipment and assist with the collection of the samples. If farm laborers are working at a test site under the direct supervision of the Principal Investigator, their names should be listed in the final report along with a statement from the Principal Investigator

that the work crew was under his or her direct supervision, was qualified to do the work, and was personally monitored to ensure that good field practices were followed. Training records, job descriptions, and curriculum vitae for the supervising Principal Investigator are certainly required. Much of this documentation can be achieved at a pre-meeting with regional personnel. The proposed content of such a meeting is described below in Section 4.

3.3 Protocol preparation

The EPA regulations for GLPs (40 CFR 160.120) provide guidelines for the preparation of a protocol. These protocol requirements do not change based on the location of the trials. The protocol should be produced by the sponsor and should carry the signature of the Study Director, his or her management, and a QA officer. For purposes of clarification, the Study Director is the individual responsible for the entire study. The Study Director will sign the final report prior to submission to the EPA. The Principal Investigator is the individual responsible for one or several field trials, usually working in the region of the trials. The Principal Investigator is responsible for the conduct of an individual trial (or trials) and compilation and submission of the trial report to the Study Director. Regardless of the location of the testing, this protocol must only be in English. The protocol must be present at the site of all field trials during all phases of conduct of the study and must be an integral part of the field report which is archived by the sponsor. Translation of protocol information is described below in Section 4. After a protocol is completed and signed by the Study Director, as with protocols intended for domestic testing, they cannot be changed except through a protocol amendment (prior to the first application) or a protocol deviation (following the first application) form signed by the Study Director and documented in the raw data. Protocol deviations can be avoided by using range extenders, such as ± 1 day, on numerical entries such as application and harvest dates in the body of the protocol. If this is done, the Study Director will be less at the mercy of the weather and equipment/personnel availability, since a protocol deviation will not have to be written if an application is delayed by a day owing to rain or unavailability of hand labor or equipment.

3.4 Test materials

If testing involves a new product that is not yet in commercial trade, the manufacturer should ship products from the same batch of the same formulation to each test location. The most recent product quality analysis and an appropriate Material Safety Data Sheet (MSDS) must accompany the product shipment. The shipping documents and the certificate of analysis for each shipment must be archived in the raw data for the report. If the residue testing is being done to register a new use for a commercial product, the Study Director must make sure that the product available in each location is the same formulation and that each different batch encountered has its own certificate of analysis to be archived along with a copy of the label and product quality analysis of the material used for the test. Material shipped in noncommercial containers must

be labeled with the name of the product, the batch number, and the study number from the protocol. The chemical must also have an expiration date and any relevant safety and storage information. The storage of the product during the course of the study and quantities used/remaining must be documented and archived. Plan to ship the test material well in advance of the study since customs regulations in most Latin American countries do not allow for quick retrieval of a shipment of an agricultural chemical. It is imperative that 'Sample without commercial value' ('Muestra sin valor comerciál') be printed on the shipping manifest. In the author's experience, the most common reason for delay of a study in Latin America is the failure of the test material to arrive and clear customs by the scheduled dates. Shipping procedures should begin well ahead of anticipated deadlines.

3.5 Quality assurance

The participation of a quality assurance representative should be an integral part of the planning process. GLP training for local personnel, site inspections by a QA agent, and audits of critical events such as applications and harvests should be programmed into the schedule for a field residue test in Latin America. Whether an in-house, corporate QA unit or a contracted QA professional is involved, scheduling potential travel dates early can avoid conflicts. Time and money can be saved if site and event audits can be programmed in a sequence that allows the QA agent to cover all aspects in a single trip rather than having to make separate trips for training, site inspections, and event audits. When planning a time/action schedule for submission of data, sufficient time must be allowed for a QA audit of the field notebooks and the final report.

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3.6 Budget considerations

The cost of a residue study in Latin America is a function of the man-hours and expenses which must be committed to the project. This is true whether the study is conducted by an international corporation or through a contract research entity.

3.6.1 Time

In the planning stage of a Latin American field residue trial, a list should be made of all personnel who will be contributing to the study. This will include US management, the Study Director, local management, Principal Investigators, agronomists and field workers, QA agents, and shipping and clerical personnel. This list is required for QA purposes as well as for budgeting.

Estimate the time, in man-hours, that each of the above will devote to the project. The man-hour costs may vary for each of the above personnel.

If the trial is planned through the services of a contract research company, their estimate should include a time and cost accounting which specifies the exact services they will perform for the trial. Services not included are the responsibility of the Study Director and sponsor. When 'contracting out' a field residue study, the contractor's cost estimate will only be a part of the total cost to the sponsor. Make sure that costs

for QA, report writing, shipping, translations, and local assistance by the sponsor are included in a budget for the project.

3.6.2 Expenses

Travel. Advance planning can keep airfare costs to a minimum. One trip by the Study Director and the QA agent to the site of each individual trial for training, pre-meetings, applications, and audits followed by a second trip to each trial site for harvests and shipping is an adequate minimum. Tropical locations in Latin America generally experience rainfall in the afternoon hours, so plan field work for the mornings and meetings for the afternoon.

Equipment. A list of equipment required for the conduct of the study should be prepared during the planning stage. This includes laboratory supplies for measuring and calibrating chemical applications, protective equipment, and shipping materials. A more complete list is given in Section 4. Once a list has been prepared, the Principal Investigators at each trial site should receive a copy. Whatever is available locally should be purchased locally, and the remainder should be shipped to the appropriate locations during the planning phase to ensure availability when required. Note that certain items of protective equipment, such as latex gloves, respirators, and spray suits, are included on a US State Department list of controlled items (22 CFR 121.1 Category X–Protective Personnel Equipment) and may be more difficult to ship (a license may be required). Allow plenty of time to deal with the bureaucracy and, of course, customs.

Crop purchase. A grower who is willing to allow a residue trial to be conducted on his or her land must be compensated for any loss of income due to the test. Using bananas as an example, the following procedure may be used to calculate the potential crop purchase expense:

1. Determine the land area required to conduct the test. If research equipment such as a mist blower or back-pack sprayer is to be used, 1 ha per trial should be sufficient. If commercial equipment such as an airplane or helicopter is to be used, 5 ha per trial for a helicopter and 15 ha per trial for an airplane may be needed. This will vary depending on the type of equipment available; the Principal Investigator or contact in the area of the trial should provide this information. Remember, also, that chemical mixing systems at commercial applicator's facilities may require more test product for adequate mixing.
2. Ask the Principal Investigator for the average yield of racemes (bunches) of bananas per hectare per month and the average price received per raceme during a normal year. The Principal Investigator should ask the grower how long they will avoid harvest owing to potential residual effects of a nonregistered chemical. The author has seen this range from 3 to 6 months in the case of bananas. With this information, a crop purchase expense can be determined for budgeting purposes.

Shipping. Crop samples harvested for residue testing are usually shipped to the USA or Europe for analysis. Shipping requires the availability of an international

shipping company such as FedEx, DHL, or PanAlpina, for example. Also required are a source of dry ice and packing materials. The shipping company can provide cost estimates. Packing materials can either be shipped to the test areas or purchased locally. The local cost of dry ice and packing materials such as coolers or ice chests should be provided by the Principal Investigator or local contact.

Analysis. The cost of chemical analyses and QA, whether carried out in corporate analytical laboratories or in a contracted facility, must be included in a budget. The costs of analytical method development, storage stability, and metabolism studies associated with specific crops should also be included, if required.

Report writing, submission preparation, and registration fees. The report writing and submission preparation are tasks to which budgets can be assigned. The registration fee for an import tolerance will depend on whether the tolerance petition is submitted as part of a larger registration package or on its own.

3.7 Communications

In addition to a possible language barrier, communications with field agronomists in Latin America has been understandably difficult, but recently the wider use of the cellular phone has made it possible to contact the field investigators while they are on location. In any case, the planning stage should include a minimum of one e-mail or voice communication between the Study Director and the Principal Investigator prior to and immediately after each critical event (applications, harvests, shipments). A majority of the problems that the author has experienced during the conduct of a foreign residue study could have been avoided had communications occurred with the Principal Investigator prior to and after significant events. The Principal Investigator must also be requested to maintain close communications with the grower to avoid problems of unscheduled chemical applications in the test plots or controls or, worse, an errant harvest of the treated plots.

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4 Pre-implementation activities

4.1 Translation of critical documents

The protocol, itself, is the most critical document, but there is no need to provide a complete translation for the principal investigator in the field, for two reasons. First, the majority of its contents may concern analysis of the samples and other items not required for conducting the field trial. Second, good field practice mandates that only one signed protocol be present during the conduct of a field study. The author has found it best to provide a two to three page 'Worksheet' which contains all information from the protocol pertinent to the conduct of the field study for use by the Principal Investigator. The worksheet should contain a disclaimer noting that if there are differences between the worksheet and the protocol, the protocol must be followed, and that the worksheet is not a protocol but rather a field guide prepared from the

protocol. The worksheet should be included in the field notebooks, and appropriate deviations or amendments which are made to the protocol must be reflected in the worksheet. The worksheet should be translated into Spanish, Portuguese, or French, depending on the location of the study. Concerning units of measurement, an English to metric 'translation' should also be made on the worksheet to ensure that conversions need not be made in the field during calibration or application events. If a protocol for a US registration includes foreign residue testing, metric equivalents should be included in parentheses following the English units (e.g., lb acre^{-1} [kg ha^{-1}]). All forms are to be completed in the field as part of the field notebook, and all forms which remain at the trial sites, such as storage, training, curriculum vitae, and archival documents, should also be translated. SOPs should also be translated since part of the GLP training requires that those who work on the trial read and understand the SOPs pertinent to the tasks performed (40 CFR 160.81).

4.2 Preparation of the field notebook formats

Electronic formats such as 'FieldNotes' which are used widely in the USA will eventually be practical for use in Latin America. Currently paper forms are the most efficient way to collect all data required under GLP for a field study. Standardized forms for such items as product receipt and storage, maintenance of equipment, calibration, applications, etc., have been designed by most major agrochemical companies for US trials. These forms can be translated and placed in chronological order of use in a field notebook starting with the test initiation form, which must be signed by the Principal Investigator and sent to the Study Director and QA agent at the time of the first application. These field notebooks can be bound in a water-resistant folder and distributed to the Principal Investigators during a pre-meeting where the use of each form is carefully explained and demonstrated. Where possible, the Study Director should be present at the first field application and should assist with the completion of the required forms. These forms will be required for each of the applications. At the first application, the trial initiation form may be completed and given immediately to the Study Director and QA agent if they are present. The Study Director should maintain a complete set of the forms in English to assist with translations.

4.3 Pre-meetings in testing regions

The first meeting in preparation for the conduct of a field residue study should include the Study Director, the QA agent, and the local personnel assisting with the study. This meeting should have the following agenda items:

1. Training course (documented) in Good Laboratory/Field Practices.
   The QA agent should make a presentation of basic GLP practices involved in the conduct of a field residue trial. If the QA agent does not speak the local language, a slide or computer presentation should be translated during the planning stage and used as 'Subtitles' for the QA presentation. Many QA agents, corporate and contract, have presentations available in Spanish and Portuguese which are

adaptable to most purposes. The message of the presentation rarely changes; good scientific practices must be followed and carefully documented.

2. Discussion of the test purpose, protocol and worksheet.
   The purpose of an import tolerance and its impact on the global registration process should be explained. The official protocol document should be reviewed in general, and practical discussion should center on the worksheet and the hands-on tasks that the personnel at the meeting will be performing.
3. Protective equipment use training and safety considerations.
   The use of proper clothing and protective equipment is mandated not only by common sense and worker safety laws and policies, but also by GLP. The GLP regulations 40 CFR 160.1(a) and 160.3(4), state that attire appropriate to the task must be worn. The type of protective equipment appropriate to the trial must be determined by the Study Director and local management. The Latin American Crop Protection Association (LACPA) is an excellent source of safety training videos and brochures in the Spanish language.
4. Equipment required for GLP testing and safety.
   In response to requests from Latin American colleagues, the author assembled what are referred to as 'GLP Kits', which are shipped to the Principal Investigator in each test region during the planning phase of the study. These kits contain the measuring and safety equipment required for the study. Providing these kits is up to the Study Director and sponsor, but the author has found that having all the needed supplies available at each test site greatly facilitates the conduct of the study. The total cost is around US $130 per kit, and the following items are generally included:

   Gloves, nitrile and latex
   Tyvek spray suit
   Respirator or dust mask
   Protective eye wear, goggles or glasses
   Graduated cylinders, polyvinylpyrrolidine (PVP), 100- and 1000-mL
   Wide-mouth bottles, PVP, 250- and 1000-mL
   Graduated beakers, PVP, 250- and 400-mL
   Plastic pipets or syringes, 3- and 30-mL
   Labels for bottles in GLP format
   Blue pens and thin-point black permanent marker
   Digital watch with stopwatch feature
   pH test strips, pH 4–9
   Anemometer, hand-held
   Max/min thermometer
   Rain gage

5. Explanation of pre-formatted field notebooks and their use.
   The two 'deliverables' from the field residue trial will be the samples, properly labeled, packed and shipped, and the field notebook, filled out correctly and completely. It is important that the Principal Investigator realize that all notations and calculations are made directly in the field notebook, not transcribed, and in ink. Multiple events, such as calibrations, applications, and harvests, must be documented on sequential individual forms. The field data in the notebook are not sent to the EPA as part of a submission package. These data must conform

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to GLPs and must be archived, but there is no requirement that the data be in English. If the forms are standardized, and a copy of the equivalent forms in English are available in the raw data archive, there is little reason to translate a field notebook unless the EPA specifically requests this as part of an audit.

6. Review of appropriate SOPs.
   Applications to and harvest of major crops such as coffee, bananas, and pineapples frequently involve procedures and equipment for which standard operating procedures have not been written. These must be identified in the planning stage, so that the Study Director or Principal Investigator can write these procedures with sufficient time to allow for review and approval. If a procedure is specific to the trial at hand, the process may be described in an addition or amendment to the protocol, but this still requires QA and management approval. In some cases, SOPs specific to a local crop are maintained at a regional site. SOPs must also be available at the site at which the raw data are archived.
7. Methodology for measurements, calibrations, applications and harvest.
   The mathematics involved with calculating the amount of active ingredient, formulated product, adjuvants, and water to put in a spray tank to achieve the application rate specified in the protocol should be addressed prior to arrival at the field for the first application. This is also true for the calibration method. The author has found that if eight agronomists are involved in a spray application, one will encounter eight distinct calibration methods. If a calibration SOP is not written for the spray equipment to be used, the precise steps in the calibration process should be documented in the field notebook.
8. Labeling, packing and shipping samples.
   Labels for residue samples should be printed out following preparation of the protocol; three labels may be needed for each individual field sample to be shipped. The first label is placed inside the bag holding the residue sample (or between the two plastic bags in a double-bagged sample). The second is stuck on the outside of the bag, and the third is placed on the outside of the box in which the sample is shipped. An assigned code, documented in the protocol, is specific for each individual sample. This code must be on each label, but it is helpful to print other information on the labels as well, such as the harvest date, location, and sample type, so that a sample can be identified in a storage facility without the presence of a protocol. This also reduces the possibility of mis-labeled samples. Packing materials include plastic bags of an adequate size for the residue sample, shipping boxes with insulation inserts, and pre-addressed shipping labels. Rather than shipping insulated boxes to Latin America, the author prefers to purchase 'coolers' (such as those manufactured by Igloo or Thermos) of an adequate size, locally. They are usually durable, have high cold retention, and can be purchased nearly anywhere at a cost generally lower than the shipping price of an insulated cardboard box. If possible, the samples should be frozen prior to packing and packed with an adequate quantity of dry ice. For a long shipment, this could be a quantity equal to twice the weight of the sample. Foreign plant residue samples shipped into the USA must also be accompanied by an USDA Plant Protection and Quarantine (PPQ) Form #597 which is entitled 'Import Permit for Plants and Plant Products', available from the USDA. Regional USDA PPQ offices at international airports can provide applications for these permits.

9. Time/action schedule and communications requirements.
   A preliminary time/action schedule for critical events, such as applications and harvests, should be prepared during the planning phase, following consultation with the regional Principal Investigators. This schedule should also include dates for QA audits, communications, and delivery of the completed field notebooks. The Study Director should try to communicate by voice or e-mail with the Principal Investigator the day before and the day after critical events. Principal Investigators, especially in remote sites, generally appreciate a Study Director who is pro-active and frequent with communications.
10. Pre-meetings with growers and cooperators.
    A pre-meeting with the grower or cooperator should begin with expressions of gratitude, but the grower/cooperator must also be made aware that their assistance is needed with several key parts of the test procedure. Some of the expectations placed on a grower/cooperator are as follows:
    - Applications of agrochemicals excluded by the protocol should not be made to the treated or control plots.
    - Records should be kept of chemical and physical crop maintenance activities in the treated and control plots.
    - Access to crop and chemical application history should be made available if possible. Several large cooperators in Latin America consider this information confidential, however.
    - Weather data at the test site, especially during the course of the study, should be provided for the Principal Investigator if available. Historical weather data are important to compare the current climate with previous years to establish that the current year's weather is not unusual or atypical.
    - Treated crops may not be used for human or animal consumption unless the product is registered locally. The Principal Investigator should confirm crop destruction procedures.
    - Only the Principal Investigator may authorize a harvest of the treated and control plots.
    - The terms of crop purchase will be negotiated prior to the first application and cannot change with market values.
    - In the author's experience, most growers and cooperators are remarkably cooperative and helpful in Latin America, and the only problems that generally arise are caused by lack of communication and misunderstandings.

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5 Implementation of testing procedures

5.1 GLP training and protocol discussion

The GLP training should consist of two parts: first, a classroom presentation of the materials described in Section 4 above, and second, a field training session where the principals of documentation of mixing, calibration, and applications can be demonstrated. Disposal, cleaning, and maintenance procedures should also be discussed and demonstrated. The classroom presentation should stress that the four key areas to successful implementation of GLPs are (1) Good Science, (2) Documentation,

(3) Documentation, and (4) Documentation! In the field training session, while achieving a successful calibration (three mock applications with a variance of less than 10%) is important, the correct documentation of this exercise is equally important, and errors, such as using pencils, performing activities not found in SOPs or the protocol, and transcribing data and calculations originally written on nonarchival papers (i.e., not in the field notebook), should be discussed and eliminated. Completion of the GLP training session should be accompanied by documentation in the archives that the course was completed and who was involved. If a 'Certificate' is awarded to the students following the successful completion of the course, it is important to stress that this is in no way a 'Certification' for them to do GLP studies. The EPA does not award or recognize 'Certifications', even though the European Union does 'Certify' laboratories and test facilities for GLP studies, as do some Latin American countries such as Brazil and Mexico. For studies submitted to the EPA, a Quality Assurance Statement signed by a qualified and independent QA agent along with appropriate audits properly documented and archived are required. If the EPA requires further assurance that the study was done under GLPs, the Agency can conduct audits at the test facilities or archival centers anywhere these studies are conducted.

5.2 Safety training

Safety training should be reviewed with the Principal Investigator at the classroom premeeting and conducted along with the field portion of the GLP training in the presence of the personnel actually performing the hands-on procedures. Both the proper use of and the reasoning behind the safety equipment provided in the 'GLP Kit' described above should be explained as both a personal safety and a GLP requirement. Many Latin American countries, such as Mexico, are currently enforcing worker safety laws.

5.3 First application

Both the Study Director and a QA agent should be present at the first application. SOPs must be followed, documentation must be correct and complete, equipment must be calibrated and its maintenance records must be documented, safety procedures must be in place, and the fine points of application methodology should be discussed with the applicator. Guidelines for applications can be found in 'Residue Chemistry Test Guidelines', Office of Prevention, Protection and Toxic Substances (OPPTS) 860.1500.

The 'treated' and 'control' plots should be of an appropriate size to yield the volume of samples required. Plots are not only correctly marked from a GLP standpoint but also flagged with sufficient warning signs to caution laborers and passers-by not to enter or harvest the plots. This is particularly important for Latin America's two largest imported crops, bananas and coffee, which tend to require longer term trials. The map of the plots made by the Principal Investigator must include distances to a fixed point in the field, such as a well or irrigation head pipe or a road intersection. This fixed point designation allows for the location of plots long after the completion of the test should this be necessary for audit purposes or for any other reason. The QA agent

should audit the first application as a critical event. In Latin America, there is a good chance that many of the people working on the study will be participating in their first GLP study. The Principal Investigator and field workers should be encouraged to ask questions of the QA agent during the course of the application, but the workers should understand that the QA agent will not interrupt an event in progress unless the integrity of the study is at risk. Following the application and clean-up, the Study Director should assemble all personnel involved with the application for a review and critique of the event with the QA agent, since probably neither will be present for any required subsequent applications.

5.4 Sampling and shipping

Sampling procedures are covered in the EPA document 'Residue Chemistry Test Guidelines' (OPPTS 860.1500, Crop Field Trials) and apply to both foreign and domestic trials. The samples to be taken must be identified in the protocol by sample number, and all samples must be tagged with this number. There is always a risk of samples being lost due to nonrefrigerated storage during a lengthy wait for customs or Office of Plant Protection and Quarantine (OPPQ) inspection or by the shipping company prior to shipment. For this reason, splitting the samples may be expedient. The protocol should state that each sample will be divided in half, while maintaining compliance with documented sampling procedures, but each half will not necessarily be analyzed. Those duplicated samples retained at the site of origin should be identified with a '. . .-B' or similar suffix. These retained split samples are held in frozen storage in the country of origin until the Study Director either receives the original portion of the samples shipped in acceptable condition, or requests that the retained 'split samples' be shipped, because the first shipment did not arrive or arrived in an unacceptable condition. Portable temperature measuring devices such as 'Hobos' which can be obtained through the manufacturer's web site (**http://www.onsetcomp.com/Products/Products.html**) can be included in each shipment. Thrifty and innovative Principal Investigators with whom the author has worked will freeze a small plastic bottle of commercial drinking water and include the frozen water in the shipment. If the water arrives completely or partially frozen or at an acceptable temperature given the stability of the chemical to be analyzed, the shipment is acceptable even if the dry ice has sublimed.

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Shipments are best made on Mondays to avoid having the samples sitting at a transfer point or in customs/OPPQ over a weekend. Availability of dry ice at the packing/shipping point must be determined by the Principal Investigator during the planning phase of the study. Field samples must be frozen after harvest within a time frame specified in the protocol. These frozen samples can then be transported covered with water ice to a location where dry ice can be obtained. Always try to take the frozen sample to the dry ice location rather than bringing the dry ice to the samples, and never transport dry ice in the cabin of a vehicle. Simple electric box freezers can be purchased anywhere in Latin America for about US $300–600, and if there is no freezer available near the test site, these simple freezers are an excellent investment. All major international shipping companies allow for tracking of samples during shipment.

6 Food Quality Protection Act (FQPA) considerations

The FQPA, which became law in 1996, is described by the EPA as follows: 'The 1996 law represents a major breakthrough, amending both major pesticide laws to establish a more consistent, protective regulatory scheme, grounded in sound science. It mandates a single, health-based standard for all pesticides in all foods; provides special protections for infants and children; expedites approval of safer pesticides; creates incentives for the development and maintenance of effective crop protection tools for American farmers; and requires periodic re-evaluation of pesticide registrations and tolerances to ensure that the scientific data supporting pesticide registrations will remain up to date in the future.' More information can be obtained on their web site, **www.epa.gov/oppsps1/fqpa**.

As an example of how this law affects residue samples from Latin America, banana and orange samples are required to be analyzed whole with the peels for tolerance setting purposes. For FQPA purposes, however, the commodity as consumed is most important. Since infants and children consume high levels of bananas and oranges as baby food and juice, the registrant may want to show that the major portion of the residues reside in the peel, because these population subgroups do not consume the peels of either commodity. From a practical point of view, the best time to separate the peel from the pulp for chemical analysis is following harvest and prior to freezing. Thus, if the commodity being tested is important in the dietary exposure analysis, the protocol should state that a second (or third) set of samples must be taken and separated into various parts to evaluate residue reduction possibilities. SOPs covering methods for removing peel from pulp should emphasize performing the separation without contaminating the pulp.

7 Reporting and closure

The guidelines for writing and submitting the report can be found in the EPA regulations for GLPs (40 CFR 160.185). The information from the Principal Investigators will appear in the appendices to the report dealing with field trial summaries. Each field trial summary should be sent to the appropriate Principal Investigator by e-mail or fax to be reviewed for spelling and accuracy prior to inclusion in the report. Once again, numerical measurements should be shown in both metric and English units.

Courtesy dictates that when a report is complete, a copy be sent to each Principal Investigator with a time estimate for submission and registration. Notes of gratitude for their participation should also be sent to the Principal Investigators, their management, and cooperating growers.

8 Abbreviations

CFR	Code of Federal Regulations
EPA	Environmental Protection Agency
FATUS	Foreign Agricultural Trade of the US
FQPA	Food Quality Protection Act

GLP	Good Laboratory Practice
LACPA	Latin American Crop Protection Association
MSDS	material safety data sheet
NAFTA	North American Free Trade Act
OPPQ	Office of Plant Protection and Quarantine
OPPTS	Office of Prevention, Protection and Toxic Substances
PPQ	plant protection and quarantine
PVP	Polyvinylpyrrolidine
QA	quality assurance
SOP	standard operating procedure

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Food processing of raw agricultural commodities for residue analysis

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1 Overview of processing of agricultural commodities

1.1 Historical background

Prior to 1985, the Environmental Protection Agency (EPA) focused mainly on the levels of pesticide residues in raw agricultural commodities at the 'farm gate', with little or no attention being given to processed foods or how residues found in raw agricultural commodities might find their way into processed food products. In addition, little attention was given to the waste products generated from food processing operations that might find their way into animal feed and ultimately into the human food chain through meat and dairy products.

Several pesticide exposure related events negatively impacted the food processing industry. The fungicide aminotriazole had a devastating affect on the cranberry industry in 1959, and the insecticides aldrin and dieldrin had similar effects on the apple and potato processing industry in the mid-1960s. Another example is the negative environmental impact of chlordane, a widely used insecticide to control wireworm in potatoes from the 1950s to 1974. Chlordane residues appeared in processed potato products, and, subsequently, this pesticide's use was prohibited by the EPA in 1974.

In 1985, regulatory attention was given to residues in processed food products as part of the data requirement for agricultural chemical registration. However, testing guidelines had not yet been established by the EPA. Once agricultural commodities left the farm gate and were received by the food processors, regulating the manufactured processed food products was under the jurisdiction of the Food and Drug Administration (FDA). Limited information was available about whether agricultural chemical residues were destroyed, transformed, or concentrated through processing.

Handbook of Residue Analytical Methods for Agrochemicals.
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The only law existing during this early period was the law entitled the Delaney Amendment of 1958. This legislation was sponsored by Mr Jim Delaney, Democratic Congressman from Queens, New York, and then Chair of the House Select Committee to Investigate the Use of Chemicals in Food Products. History was made when this law called for 'zero cancer risk' in our food supply. The Delaney clause-amendment became part of Section 409 of the Federal Food, Drug, and Cosmetic Act of 1958. In reality, the restrictions that grew from the Delaney clause, strict though they were in the beginning, only worsened during the following years. At the time the law was passed, scientists were able to detect the presence of chemical compounds in food at levels of several parts per million (ppm). However, over the next four decades that the Delaney Clause reigned, researchers were able to detect smaller and smaller concentrations. Detection of residues at the one part per billion (ppb) level became common, and even detection at parts per trillion (ppt) is now possible. Recent scientific advances showed that the Delaney specifications were impractical, and regulations and guidelines need continual updating in order to keep up with the newest technology.[1] As the analytical technology improved, the Delaney clause became an unrealistic goal without scientific basis.

To understand better the fate of agricultural chemicals in processed foods, the EPA now requires that processing studies be carried out on many agricultural raw commodities. EPA requirements are guided by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) of 1972, the Federal Food, Drug and Cosmetic Act (FFDCA), and their amendment, the Food Quality Protection Act (FQPA) of 1996. Other laws that also impact the EPA pesticide regulation include the Safe Drinking Water Act (SDWA), the Federal Advisory Committee Act (FACA), and the Freedom of Information Act (FOIA).[2]

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The primary focus of FIFRA is to provide federal control of pesticide distribution, sale, and use.[3] Under FIFRA, the EPA was given authority to study the consequences of pesticide usage. FIFRA requires that all pesticide uses in the United States be registered by EPA. Registration assures that pesticides will be properly labeled and that if produced and used in accordance with specifications, pesticides will not cause unreasonable harm to the environment.[3]

The FQPA is another important statute for regulating pesticides. The FQPA amendments to FIFRA changed the way EPA regulates pesticides. The requirements included a new safety standard – reasonable certainty of no harm – which must be applied to all pesticides used on foods.[4]

The FFDCA governs the establishment of pesticide tolerance for food and feed products. A tolerance is the maximum level of pesticide residues allowed in or on human food and animal feed.[2]

1.2 Basis for selecting a process method

Processing methods are based on the food industry's best practices. The basis for selecting a particular processing method depends upon the commodity and the final processed fractions desired. As illustrating examples, several general processing methods for selected crops are presented below. This information provides a better understanding of the physical and biological processes that will affect both the chemical

nature and the magnitude of pesticide residues during crop processing. Recognizing that individual food processing companies may use different procedures to make their individual products is important. However, whatever procedures are used, they must represent the processes involved in commercial operations, and the fractions produced must be representative of those found in commerce.

1.2.1 Grapes

Grapes may be used for making food products and for making wine. Grapes are first ground and pressed when being made into juice, jams, or jellies. The juice may be further filtered prior to making juice or jelly. If juice is made, unclarified juice is often held at refrigeration temperatures for extended time periods to allow precipitation of tartaric acid salts and then filtered. The press pulp is usually discarded in landfills or fed to animals. Jam production requires that at least some pulp be retained in the juice to meet the proper quality attributes (e.g., texture). Heating is used during the processing of jam and jelly.

If the grapes are destined for use in wine production, no thermal processing steps are involved. The grapes are ground, and the entire mass is fermented. The fermented juice is then decanted from the pulp, or lees, and moved into other tanks for aging. In some cases, a secondary fermentation is encouraged. The wine is then racked or filtered to remove yeast cells and other solids and bottled.

1.2.2 Tomatoes

Tomatoes are washed upon receipt at the processing plant. Canned tomatoes are peeled by dipping the tomatoes in boiling water or lye or by steam treatment and cut for the size of product to be canned. Tomato juice is added to the cans before closing. They are heated in a retort to achieve commercial sterility.

For juice, purée, and paste, the tomatoes are crushed, heated to inactivate enzymes, and pumped through a finisher where the juice is separated from the pulp. The pulp is fed to animals. The juice is canned and is also evaporated to make purée and paste. All of these processes require heating.

1.2.3 Corn

Corn may either be dry milled to produce grits and flour fractions or wet milled to produce corn starch and gluten fractions. For dry milling, a small amount of water is added to the corn, and the germ is removed. The remaining material is ground and sifted to obtain various fractions from coarse grits to flour. For wet milling, the corn is steeped with water to increase its moisture content and to soften the corn. The corn is then ground, and the germ is removed. The remaining slurry is further sheared, and the gluten and starch are mechanically separated and dried.

The germ fraction contains the oil. The oil is either recovered by pressing or by solvent extraction. In both cases, the oil is further refined by mixing the oil with clay materials to absorb color followed by mechanical separation to remove the clay. The oil is deodorized under high temperature and low vacuum followed by removal of high melting point fats by refrigeration and filtering.

1.2.4 Sugar

Sugar (sucrose) is obtained from either sugar beets or sugarcane. Sugar beets are traditionally diffused with water to extract the sugar from the pulp. The sugar is then crystallized, mechanically separated, and washed to produce white sugar.

Cane sugar is processed using one of two methods of extraction. One method uses diffusion and the other uses roller press extraction. Both methods may be used to make sugar and molasses fractions from sugarcane. However, roller press extraction is used by a greater percentage of the industry and, therefore, is the better method for a small-scale study.

Sugar made from either sugar beets or sugarcane is a perfectly acceptable product. However, the molasses produced from these crops is quite different: molasses from sugar beets is an animal feed product, whereas molasses from sugarcane is marketed as a human food known as blackstrap molasses.

1.2.5 Citrus

Citrus (oranges, lemons, grapefruit) is processed into juice and oil for human uses and into molasses and dried pulp for use as animal feed. The fruit is first washed with a detergent and rinsed with water. The oil is localized in oil sacs on the surface of the fruit. The surface is scarified under a water spray to form an emulsion of oil and water. The oil is recovered by centrifugation, filtered to remove high melting point fats, and dried with sodium sulfate, which is removed by filtration.

The juice is recovered by inserting tubular knives into the fruit and squeezing the fruit. Alternatively, the fruit may be cut in half and squeezed. The recovered juice is put through a finisher to remove a portion of the pulp sacs, is heated to inactivate enzymes, and is either bottled as single-strength juice or concentrated for frozen concentrate.

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The pulp is ground, neutralized with lime, and pressed. The press liquor is concentrated to make molasses for animal feed. The pressed pulp is dried and used as an animal feed.

2 Laboratory/pilot processing of agricultural commodities

2.1 Processing requirements of individual agricultural commodities

Given the requirements of the laws and the resulting regulations discussed above, scientific testing protocols are designed in accordance with ‘real-world’ processing procedures as closely as possible. A sound knowledge of processing procedures used by the food industry is essential in determining the processing requirements of individual agricultural commodities. In addition, interaction with members of the food processing industry helps to ensure that small-scale processing practices are in line with current industry best practices.

The required processed commodities from various crops are specified by the EPA. Those requirements have changed periodically over the years. Therefore, the authors recommend that the reader consult with the requirements listed in Table 1

of the EPA document 'Raw Agricultural and Processed Commodities and Feedstuffs Derived from Crops' (EPA's Residue Chemistry Test Guidelines published in August 1996, Section 860.1000, Background, EPA publication number 712-C-96-169) or other EPA documents that list the processing requirements for various crops.[5]

2.2 Pilot laboratory processing versus commercial processing

The food industry in the USA has developed large-scale production practices for commodities. Most agricultural crops are processed on a continuous basis in large production plants. For example, tomatoes are typically grown and become ripe from June to October. During this time, they are harvested and transported by truck to centrally located processing plants that operate 24 h per day, 7 days per week from the beginning to the end of harvest. The equipment is designed to handle tons of tomatoes per hour. Under these conditions, a steady-state operation is attained soon after production begins and is maintained unless interrupted by equipment failure.

By contrast, processing in a pilot laboratory is essentially a batch operation. Small quantities of crop materials are transported to the laboratory and put through each unit operation. Steady-state conditions are seldom achieved because of the short duration of processing time. Since most food processing equipment is started and stopped with water and the raw agricultural commodity (RAC) is introduced after the equipment is up and running, there will be some dilution of the processed commodity with water, perhaps to a greater extent than in a large production plant. Even with these differences, well-designed and executed small-scale operating procedures will produce commodities that represent commercially produced foods.

2.2.1 Adapting a commercial process to a controlled and well-documented pilot plant process

The first step in adapting a commercial process to a pilot process is to understand the sequence of, and reasons for, the various unit operations in a commercial process. Food processing involves three basic fundamentals: chemistry, microbiology, and engineering. Food production is composed of a series of unit processes, each designed to accomplish some change in the food. Each unit process may cause chemical changes to a food that may affect the color, the flavor, or the physical characteristics of the commodity. The unit process may also be a simple separation of food components that minimally affects the chemistry. The duration of the processing and the temperatures involved affect the microbiology and, therefore, the safety of the food.

The sequence of unit processes is vitally important in achieving the desired end products.

2.2.2 Sources of processing information

The engineering component of food processing procedures is critical. Proper equipment that allows the simulation of commercial processing must be used. Each

step in the process sequence is conducted to produce the desired change in the food and is accomplished with machines specially designed for the intended process. The commercial machines must be understood and mimicked on a laboratory scale for successful simulation of commercial practices. This is often a limiting factor for processing laboratories, because the necessary machinery can be capital intensive and is usually designed for high throughput rates and volumes. A thorough understanding of the process step may allow less expensive procedures to be adapted to achieve the same food product as achieved in commercial production.

The literature is a rich source of information related to food products and processing. Journal articles provide extensive information, although the chances of finding an exact 'how to' process for converting an RAC into an EPA-required processed commodity are minimal. Most journal articles deal with in-depth research and discussion of a single topic in the process or related to the product. An experienced food technologist could piece together information from several sources to define a process.

Members of academia in the agricultural and food chemistry areas are often helpful in defining a process for a particular crop commodity. Many members of academia, specialized in conducting research on one crop, such as corn, olives, or tomatoes, have developed a good deal of experience and are often willing to share their expertise with others.

Food processing companies, although an excellent source of information about processing RACs, often wish to protect their own processing procedures as proprietary information. These processes may provide them with an economically competitive edge. Therefore, they are reluctant to share information with outsiders, especially if they do not know the people making the inquiries. If they are approached, however, by people who can position themselves as noncompetitors, some industry members are willing to discuss generic processing procedures. In reality, since the purpose of the crop processing study is to simulate commercial practice, generic procedures are really those that should be used.

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Consultants who were members of a particular segment of the food processing industry may be very instrumental in developing the best processing practices and describing them in a standard operation procedure (SOP). Consultants are often able to develop the necessary information for an SOP quickly.

Equipment and ingredient suppliers are an excellent source of information about food processing procedures. Since the processing companies use their products, they have worked extensively with many of the food processors and are very familiar with the technology. The goal of equipment and ingredient suppliers is to sell more of their products, so they will often share information with the knowledge that the information may lead to additional sales in the future.

Trade associations are often a good source of information. If they cannot answer questions directly, they may be able to offer information for making contacts in the industry. There are numerous trade associations on both a national and local level in the USA. They include the Snack Food Association, the National Food Processors Association, the Northwest Food Processors Association, the California League of Food Processors, the California Grape Commission, and numerous others.

2.2.3 Simulating commercial production on a small scale

Commercial production of food products is conducted in large-scale processing plants. As already mentioned, tomatoes are processed in plants continuously from June to late fall (autumn), 24 h per day, 7 days per week. This is also true of potatoes, wheat, soybeans, corn, and other large-scale commodity crops. Some crops, such as grapes, that might have a relatively short harvest season, may be processed around the clock to generate intermediate products, such as juice or young wine that can be stored for extended periods of time, whereas final products, which can be made from the intermediate products, may be produced over time as consumer demand requires. Final products may also be stored in their final retail or food service containers until demand requires distribution.

The amounts of raw agricultural commodities available to a laboratory for processing are very small. Maintenance of sample integrity and identity during large-scale continuous commercial production is impossible; therefore, the development of small-scale batch sample processing alternatives is necessary to accommodate laboratory food processing studies. The laboratory, small-scale batch method of sample processing should be designed to simulate commercial large-scale operations as closely as possible.

The key to simulating commercial large-scale production with small-batch processing is to simulate each process step accurately. Each process step in large-scale processing is operated under controls to maintain flow rates based on equipment sizes, temperatures, pressures, and other appropriate control factors to obtain the desired targeted processed products. For example, one step in the processing of tomatoes is a heating step of freshly comminuted tomatoes to inactivate pectinesterase, an enzyme that will cause viscosity reduction of purée and paste. Whether in a large- or small-scale processing scenario, attaining the correct temperature as soon as possible after comminution is a critical step to the process.

Another significant difference between large- and small-scale processing is dilution of the product samples with water. Food processing equipment for fluids often needs to be started with water, and the food needs to be flushed from the system with water before the process is shut down. When making small batch samples in this type of equipment, care must be taken to obtain a representative sample with a minimum of dilution. There will also be a difference in the weights of the sample into and out of the process due to water addition or sample loss to minimize dilution.

In the initial phases of commercial production, many agricultural commodities are graded into size or color ranges. Fruit of a given size may go to juice, purée, or some other product, while fruit of a different size may go to whole or sliced fruit products because they fit into the can or another container efficiently. For small-scale processing of discrete batches, all of the crop sample must be used, and sorting by size is not typically done. As a result, processing may be more difficult because the processing equipment has to handle various sizes rather than a narrow range of sizes. While this typically does not prevent processing of crop samples, the processing effort may be more challenging.

2.2.4 Impact of raw material quality

Raw material quality is an important factor when processing raw agricultural commodities. Crops are commercially harvested when they are at the optimum maturity for processing. Processing tomatoes are harvested when they are fully ripe but not so soft that they will be crushed in the gondola trucks during transportation from fields to the centralized processing plant. The number of green tomatoes must be minimized because they do not produce sufficient quantities of juice for juice, purée, and paste production. If canned whole tomatoes or dices are desired, green tomatoes will be too hard, and over-ripe tomatoes will be too soft. Improperly matured crops will also significantly impact yields in the production plant and adversely impact the economic value of production.

Most experimental protocols determine the time of harvest from the application dates of the experimental chemical product. If the study's Principal Investigator has guessed correctly months earlier when the first application was made, the crop will be at the proper maturity when harvested. However, if the weather or some other factor changes, the crop may be immature or over-ripe. If the experimental protocol does not permit for these variations in growing conditions, there may be an adverse impact on the ability to process the test samples properly, possibly resulting in atypical processed fraction samples.

Exaggerated chemical application rates may also adversely affect the crop quality. If the exaggerated rate causes the crop to be abnormal in some way at maturity, the processing applied to the crop may not result in typical fraction samples. The adverse impact may be obvious, such as slowed maturation of the crop, or the impact may be less obvious, such as a change to the internal structure of the individual fruits and vegetables, grains, nuts or roots.

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Sample containers used to transport the crop to the processing laboratory can also affect the quality of the crop prior to processing. All crops when harvested are still living specimens. They continue to respire and mature. Crops should not be put into tightly closed containers, such as plastic bags, that do not allow moisture and ethylene oxide from respiration to escape. Fruits and vegetables packed in plastic bags will become warm and moist on the surface, mold will grow, and maturity will advance more rapidly. Fruit will become soft in only 2–3 days in closed plastic bags. Delivery of samples directly from the field trial plot to the processing facility is the ideal situation because closed plastic bags can be avoided, and the crop can be placed under refrigeration with sufficient air circulation to permit rapid cooling and slowing of the maturation process.

Delivery of field trial commodities directly to a processing laboratory is not always possible or convenient because great distances may be involved; shipment by overnight express becomes necessary. In such instances, field trial samples may have to be packed in plastic bags, preferably after cooling the crop to remove field heat. Packing in plastic bags may be necessary to prevent any leakage from containers on to the carrier's equipment; leakage could result in delays in delivery. Keeping the weight of the commodity placed in each package to less than 25 lb (ca 11 kg) reduces the crushing potential during the multi-handling by the carrier's personnel. Upon delivery to the processing laboratory's coolers, the containers should be opened to allow the crop to breathe and to allow rapid cooling to take place. In some instances,

the samples may have to be removed from the shipping boxes and placed into tubs for even faster chilling and slower maturation before processing.

2.2.5 Factors influencing raw material quantities

The study protocol determines the quantities of raw materials to be processed. The protocol should state the quantity of each fraction sample to be provided and whether multiple samples are to be drawn from each fraction sample. The wording of the protocol should allow for a range of quantities that is to be provided for each processes commodity to avoid having to prepare protocol deviations. For example, stating that a minimum of 1 lb of purée is to be provided avoids a protocol deviation if the processor provides 1.1 lb of purée.

The smallest available sample fraction for a crop is a second major influence that determines the quantities of raw materials to be processed. For a given processing scale, perhaps 300 lb of tomatoes are needed to obtain the required processed commodity quantities. However, oranges typically required 800–1000 lb for each sample because the limiting factor is the orange oil. Navel oranges provide approximately 4–5 lb of oil per ton of oranges, and Valencia oranges provide approximately 6–7 lb of oil per ton of oranges. These quantities of oil result from finely tuned commercial production processes. Therefore, 800–1000 lb of oranges will typically provide 1–1.5 lb of orange oil on a small-scale processing basis.

A third factor affecting the quantity to be processed is the scale of the processing operation. A laboratory-scale operation will typically require less sample than a pilot-scale operation and much less than a commercial scale operation. Throughout the process, each unit operation must be supplied sufficient material to operate the process adequately while providing representative samples from the process.

2.3 Effect of processing on pesticide residues

Residues on crops may either be on the surface of the crops if applied as surface sprays, or they may be throughout the crop matrix if applied as systemic treatments. If the product is applied as a spray, the residue may be removed or significantly diminished as a result of washing. If the crop is not washed, e.g., grapes used in wine, juices, or raisins, the residue may remain with the fruit.

If the treatment is a systemic application, the chemical will not be removed by washing. The residue may be affected, however, by processing procedures such as evaporation or use of high temperatures. Evaporation and other forms of physical removal of water, such as pressing and drying, separates fluids from solid components. Water removal by evaporation and drying makes a fairly specific separation of water and solids, whereas pressing still carries soluble solids with the water. If the chemical compound is volatile, the residue may go with the water. If not, the residue may be concentrated with the solids. In the case of pressing, some of the residue may go with the fluid, and some may stay with the solids.

Heating of foods may be moderate, such as pasteurizing at temperatures of 65–88 °C, intermediate, such as thermal processing (canning) at temperatures of 88–121 °C, or high, such as frying at approximately 177 °C or oil deodorizing at

177–230 °C. Depending upon the heat lability of the compound, the residue may or may not be affected.

3 Good Laboratory Practice (GLP) regulations and their impact on the small-scale processing procedures

3.1 Development and validation of SOPs

Agricultural chemical companies conduct experimental research on new products to provide data to the EPA for registration of their products. Both the EPA and the agricultural chemical companies require that the data represent actual use conditions as closely as possible. The goal of small batch processing of samples is to simulate typical commercial practices as closely as possible. Therefore, SOPs that reflect commercial practices should be developed to meet the GLP specification.

An understanding of commercial food processing is extremely important in developing representative SOPs. The uses of the SOP developer's own background may be all that is necessary for some processing procedure development. However, when further information is necessary, one can rely on a number of sources. The literature, academia, food processing companies, consultants, equipment and ingredient suppliers, and food industry associations are all sources of information as described earlier. All SOPs should be challenged and validated before using them in actual pesticide residue testing studies.

SOPs should outline the steps that are required to carry out the processing of an RAC to provide specific processed commodities and should enable others to repeat the work. At the same time, since food crops and ingredients may vary, the SOP needs to provide enough flexibility that variations in processing can be accomplished to accommodate those crop differences without generating GLP deviations at every turn. Raw data notes should complement the SOPs and provide sufficient detail to allow the study to be reproduced in the future if needed.

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The processing specified by each SOP would vary depending on the raw agricultural commodity and the required processed commodities. In general, most processing will begin with cleaning steps that may include washing, foreign material removal, or some other initial purification of the raw material. Separating components (e.g., grapes from stems), peeling or dehulling, soaking, or comminuting will follow this cleanup step. The next step may include separation of component parts of the fruit, vegetable, nut, or grain followed by further purification steps. Alternatively, the product may have been finished at that point and placed in a container for final processing that may include thermal processing, refrigeration, or freezing. In all cases, the final step for pesticide residue processing studies is freezing in order to preserve the samples for chemical analyses of the residues of concern.

3.2 Development of processing protocol

3.2.1 The role of the protocol

Any residue study conducted under GLP is required to have a protocol written by or under the supervision of the Study Director. The protocol contains instructions

for the conduct of the study and identifies the responsible parties for each phase of the study. The processing phase of the protocol may be contained within the body of the protocol or may, at times, be added as an amendment when the processing details are not known at the time the protocol is prepared. A copy of the processing phase of the protocol, signed by the Study Director, must be in the possession of the processing facility before any work on the study can proceed.

The processing phase of the protocol will identify the site of the processing and will identify the individual at that site responsible for the conduct of the study. The individual responsible for the processing phase of the study is generally referred to as the Principal Investigator. The Principal Investigator will conduct the processing study under the GLP standard set forth in 40 CFR Part 160, in observance of the requirements set forth in the protocol and within the parameters of the SOPs of the processing facility. In the event that an SOP is in conflict with the direction of the protocol, the protocol will take precedence. Typically, the protocol will provide a broad outline of the study, whereas the SOPs contain specific details of the process.

The protocol will provide important date and time requirements. Estimated start and completion dates for the study are required by regulation. In addition, the Study Director may specify a maximum storage time of the RAC prior to processing if the chemical in question or its metabolites degrade rapidly. A maximum time after generation of a processed fraction before that fraction is placed into frozen storage may also be specified. A maximum storage time prior to shipment may also be specified.

3.2.2 Processing study work flow

At a minimum, the processing phase must identify the RAC to be processed and the processed fractions to be produced. Other essential information is the quantity of the RAC to be delivered to the processor and an indication of the quantity of the processed fractions to be produced. The minimum quantity of each processed fraction is driven by the requirement of the analytical laboratory and in most cases includes a substantial excess allowance. The minimum quantity of RAC to be processed may be driven by the amount of processed commodity to be produced or the minimum raw material requirements of some processes or equipment to be used. Another factor to be considered in establishing minimum amounts of both RAC and processed fractions is the amount required for a representative sample. An amount of 10 lb of strawberries may provide a representative sample of an experimental plot, whereas the same amount of pumpkins almost certainly would not.

Specific information on the handling of the processed fractions may also be included. Specific containers or types of containers may be required to minimize analytical interference. Sample identification numbers may be assigned in the protocol or may be generated by the processing facility. In either case, each processed fraction should have a unique identification number to reduce confusion at the processing facility and at the analytical laboratory where the residues will be determined.

Typical storage requirements for the finished fractions will be specified by the protocol. Shipping instructions should identify an acceptable carrier and temperature and condition of transport, e.g., frozen. If dry-ice shipment is recommended, a minimum amount of dry-ice per container should be indicated. An address for shipment of the

finished fractions and shipping instructions should be contained in the processing phase of the protocol.

3.2.3 Selection of processed fractions for analysis

The rationale for producing processed fractions for residue analysis is that a pesticide which may be undetectable in an RAC may be concentrated in one or more processed fractions of that commodity to a level of regulatory concern. A good example is orange oil that is recovered at levels on the order of 0.1% of the total weight of the fruit. The oil is recovered only from the outer surface of the orange, and a pesticide applied to the surface of the orange may be removed with the oil, resulting in a very high concentration factor.

As described previously, the EPA identifies processed commodities of interest in Table 1 of the Series 860 Residue Chemistry Test Guidelines.[5] The interest is in processed commodities that provide a potential for concentration of a pesticide and in byproducts of the processing that are used as major feedstuffs for food producing animals. Processing byproducts that are used to feed animals represent a human food safety risk through the potential for biological accumulation in milk, eggs, or meat. An organization interested in submitting a chemical for registration on a particular crop would need to provide residue data on the RAC and on any processed commodities and feedstuffs listed in the Series 860 Table 1.

In addition to the processed commodities listed in the Series 860 Table 1,[5] some registrants choose also to provide data on other processed fractions. With the advent of the Food Quality Protection Act of 1996 and the emphasis on protecting the food supply for children, collecting residue data on additional processed commodities may be prudent. Fruit purées are popular as baby food in the USA and are an example of a processed commodity that could come under additional scrutiny since they make up a large portion of an infant diet.

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Waste water from the processing operation is another example of a fraction that may be analyzed for residues if there is any question of environmental risk from processing plant waste water disposal.

The registrant may submit residue data on any number of processed fractions. Table 1 of the 860 Series[5] just defines what the EPA is required. A registrant may be wise to take a proactive approach and collect residue data on additional processed commodities for use in dietary exposure assessments.

3.3 Role of study personnel

The Study Director is the pivotal person in any GLP study. The sponsor initiates the study and assigns a Study Director to act as the primary control point for all aspects of the study. The Processing Principle Investigator (PPI) acts as an agent of the Study Director and handles the processing phase of the study. The PPI reports directly to the Study Director, and quality assurance (QA) documents resulting from the processing phase are sent to the Study Director for approval. The Study Director has final say in all questions of compliance with GLP and interpretations of the protocol. The

PPI acts as a resource to the Study Director with specific expertise in the area of food processing and acts as an extension of the Study Director in supervising of the processing phase of the study.

The Field Principal Investigator (FPI) provides the same services to the Study Director as the PPI except that the FPI's services are provided in the area of growing crops and application of pesticides. The FPI and the PPI must interact so that information on application timing and its impact on harvest date and subsequent delivery of the RAC to the processing facility is communicated in a timely fashion. Delivery method, RAC condition, and timing are all important aspects of the processing phase that are actually controlled by the FPI.

3.4 Protocol deviations

The GLP standards identify three types of deviations: there are deviations from GLP standards, from the protocol, and from SOPs. Any deviation must be reported to the Study Director and documented in the raw data notebook. There are differences in the reporting process for the types of deviations.

Protocol deviations in the processing phase of the study must be reported to the Study Director without delay. The Study Director will determine any potential impact upon the study that would result from the protocol deviation and will advise the PPI how to proceed with the study. Regardless of the form of communication by which the Study Director is notified of the protocol deviation, a formal description of the protocol deviation must be written by the PPI and submitted to the Study Director for an assessment of impact on the study. The assessment of impact by the Study Director should address any scientific and GLP compliance issues. A signed copy of the deviation report is included with the raw data notebook.

GLP and SOP deviations are formally written up and signed by the PPI for inclusion with the raw data notebook. The PPI can assess the impact of the SOP deviation on the study. If there is no impact to the scientific or compliance aspects of the study, the signed original SOP deviation description is included in the raw data notebook. The Study Director is notified of the no impact SOP deviation as a matter of courtesy. If the PPI determines that there may be an impact on the study, the Study Director must be consulted promptly to determine the proper course of action. Any impact on the scientific aspect of the study resulting from an SOP deviation may generate a protocol deviation. Any impact on the GLP compliance of the study resulting from an SOP or GLP deviation will at the very least need to be addressed in the GLP compliance statement from the processing facility which is included in the raw data notebook.

4 Organization of a processing report

4.1 Raw data notebook

The raw data notebook is normally submitted separately from the processing report. The information in the raw data is used to compile the processing report. All data

generated during the conduct of a study should be recorded directly, promptly, and legibly in ink. All entries should be dated on the day of entry and signed and/or initialed by the person entering the data.

The processing study raw data process notebook may include the following information:

- title page
- GLP/protocol/SOP deviations [if deviation(s) occur]
- sponsor protocol amendments (when provided)
- sponsor protocol (the key document that is originated by the Study Director)
- memos to the raw data notebook (documents to clarify information in the raw data, when necessary)
- personnel engaged in the processing study (a listing of individuals involved in the conduct of the processing study)
- process flow diagrams (a flow diagram outlining each step of processing)
- process notebook error correction procedure (an SOP detailing the codes used in the correction of errors in raw data)
- sample receipt form (a form that documents receipt of the samples by the processing laboratory)
- receiving documents (when provided) (chain of custody document that accompanies the sample)
- untreated and treated sample storage activity log (documentation of sample movement in and out of storage)
- copies of pertinent sample storage temperature charts and logs (documentation showing temperatures maintained during sample storage)
- process data sheets for untreated and treated samples (documents containing all raw data collected in the course of processing the sample)
- shipping documents (chain of custody and shipping documentation of processed sample fractions).

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Note. The nature and special requirements of a given processing study may require a different format than that outlined above.

4.2 Summary report of processing procedures

The processing report contains a summary of the processing data. An outline of each report element is presented in the following sections with clarifications added as needed. The processing report may be included as an appendix in the final report of the processing study to satisfy the FIFRA GLP Standards 40 CFR 160.185(a)(12).

A suggested format is presented below. The format may be different depending on the requirements of the sponsor or the procedures of the processing laboratory.

1. Title page. This page is numbered as 'Page 1 of __'. The following is the information that may appear on the title page:

   (a) report title (usually contains the title of the protocol; the title includes the name of the chemical and test system being examined)
   (b) study sponsor
   (c) testing facility
   (d) Study Director: the person who is ultimately responsible for the total conduct of the study
   (e) processing test site: includes the name and address of the processing laboratory responsible for the processing phase of the study
   (f) author: the name of the PPI
   (g) report date: the date on which the report is written
   (h) test system: identifies the commodity processed
   (i) protocol/study number: a number assigned to identify the study that will identify all aspects of the study through all conduct phases from start to completion.
2. GLP compliance statement
   (a) a statement that the processing phase of the study was conducted in compliance with the 40 CFR Part 160 FIFRA; GLP standards; final rule
   (b) this page is signed and dated by the PPI.
3. Table of contents
4. QA statement
   (a) this page lists the dates and type of inspections conducted by the QA unit in addition to the dates the inspection reports were submitted to the Study Director, sponsor management, and processor management
   (b) this page is signed and dated by the QA specialist
5. Record of transfer and retention statement: this statement lists the specific raw data included in the study and where these data are archived.
6. Processing summary: this section may consist of a general paragraph describing the commercial process and the specific variety received as the test commodity. The processing summary will continue with a step-by-step description of the processing laboratory operations required to simulate the production of the required fractions.
7. Processing technology references: this is a list of the commercial process references used to describe the commercial process conditions used in the development of the laboratory scale SOPs.
8. Equipment: a listing of the equipment used in the laboratory processing of the RAC.
9. Personnel: a listing of the personnel who participated in the conduct of the study.
10. Material balance or weight distribution sheets: the laboratory process flow diagrams with material balance or weight distribution sheets for each test system sample are summarized from the raw data process data records. Material balance refers to the balance of a particular component of the food, usually solids, throughout a process. If moisture or total solids are not analyzed for each process stream and only the weights of each process stream are recorded, then the term weight distribution is more properly used.
11. Chain of custody: this page includes the receiving date and time and condition of storage of the samples prior to processing and the condition the processed fractions were maintained at prior to shipping.

5 Summary

The evaluation of pesticide residues in the processed commodities of key agricultural crops is an important component of the food safety evaluation completed by EPA prior to granting a marketing license to a pesticide manufacturer. The preparation of these samples and subsequent handling have a major impact on the quality and integrity of data supplied to the EPA to be used in their review process. Close adherence to the handling, processing, and data reporting procedure outlined in the preceding paragraphs will ensure that studies are conducted in a cost-effective manner and that the data produced as a result of these studies will ensure a quality decision by the EPA. The research and testing activities outlined in this article form an integral part of the food quality protection effort that ensures a safe food supply for consumers in the United States.

References

1. E. Holland, The Delaney Era Ends, 1997 Frontiers, A Chronicle of Cancer Programs at The Ohio State University, Ohio State University, Columbus, OH (1997).
2. United States Environmental Protection Agency Web site: **www.epa.gov/pesticides/citizens/legisfac.htm.**
3. United States Environmental Protection Agency Web site: **www.epa.gov/region5/defs/html/fifra.htm.**
4. United States Environmental Protection Agency Web site: **www.epa.gov/oppfead1/fqpa/.**
5. EPA Residue Chemistry Test Guidelines OPPTS 860.1000, Background, United States Environmental Protection Agency, Washington, DC (1996).

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Best practices in the implementation of a large-scale market basket residue survey study

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1 Introduction

1.1 General considerations

Large-scale market basket surveys (LSMBSs) are studies carried out specifically to obtain information at the consumer level. The findings of such a survey are intended to support regulatory or business objectives concerning items purchased and used by consumers. The type of information collected might, for example, consist of levels of pesticide residues or nutrients in foods or of plasticizers in personal-care products. The items examined during the survey can include any type of product with which a consumer comes into contact, such as packaged hand lotions, raw or processed foods, or toys and teething rings. Data generated in market basket surveys are commonly applied in the assessment, management, and mitigation of risks (dietary and otherwise), in addressing public concerns, and in product stewardship. In general, a large-scale market basket survey

- involves a large number of exemplars (individual consumer items)
- extends over a specified geographical area
- lasts over a defined time period
- features several analytes or properties to be determined.

LSMBSs are designed so that the types of data to be collected meet particular objectives regarding how the data will be used. For example, the number of exemplars collected in the LSMBS will depend on the use planned for the study results. Thus, if the levels of plasticizer in packaged foods will be determined to address an acute (i.e., short-term) dietary exposure and risk concern, the sample size (i.e., number of food packages to be collected) must be adequate to support the higher percentiles

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of the residue distribution. Conversely, an issue of chronic (i.e., long-term) concern will focus on mean residue levels and exposures. Therefore, if the issue is chronic exposure, the sample size does not have to be as large as for the acute case, because the upper end of the tail of the distribution does not have to be as well defined.

Considerations in designing a market basket survey also include the period of time over which product samples will be collected. For example, should samples be collected over one year to illustrate an annualized perspective on the data of interest, or is the issue more aptly addressed with a shorter collection period such as one season or one month?

The design of the survey must address where samples should be collected. There are two parts to the 'where' component of this point. The first 'where' component addresses the geographic location for sample collection, and the second 'where' addresses the type of retail outlet. In other words, products might be collected across the USA, or collection could be confined to one state or geographic region. Products might, for example, be collected from grocery stores, convenience stores, and superettes or only from supermarkets.

All of these considerations, and others, must be examined from a number of perspectives in preparing the design of the LSMBS. First, each factor must be considered statistically. Second, each must be considered in the scientific context in which the data will be developed. For example, the sensitivity of the residue analytical method needs to be kept in mind if analyses will be required. Third, each factor must be considered in the scientific context in which it will be applied, such as in risk assessment or addressing public concerns. The third factor controls the selection of the products to be investigated and the properties to be determined. Fourth, the logistics for conducting and executing the study must be considered. This point includes such items as collection of product exemplars, transfer of the products to laboratories, and generation of analytical data, with appropriate documentation at all stages. Coordination, planning, and communication are, therefore, key components to the success of an LSMBS.

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1.2 Case study (Organophosphates Market Basket Survey)

A comprehensive, large-scale market basket survey, the Organophosphates Market Basket Survey (OPMBS), was conducted during 1999–2000 to determine the magnitude of residues of 22 organophosphate insecticides and their metabolites in 13 types of fresh fruits and vegetables. The study was successfully completed, and all study objectives were met. Thus, the OPMBS serves as a case study to illustrate key aspects of the planning and conduct of an LSMBS, in general.

The OPMBS was sponsored by a task force, consisting of major registrants of organophosphate pesticides, and utilized three contract organizations to carry out study management, design and conduct of sample collection, and quality assurance (QA). Four analytical laboratories performed the necessary residue analyses.

Study design was a joint effort of the task force and the three contract organizations, with input from the laboratories on analysis-specific issues. The design is described briefly below. Specific issues regarding the design and the conduct of the OPMBS are discussed in detail in the following sections.

During the OPMBS, up to 500 samples of each of 13 different fresh commodities (apples, oranges, broccoli, peaches, strawberries, green beans, sweet corn, potatoes, cherries, tomatoes, cucumbers, grapes, head lettuce) were collected over a 1-year period. The sampling regime followed a statistical design, involving purchase of commodities across the continental United States. Commodities were collected from specified supermarkets, selected from a database of 96 400 supermarkets, superettes, and chain convenience stores. The selection process used a set of pre-established criteria that were incorporated into the statistical design. These criteria included the geographic location of the store, the size of the store in terms of sales volume, and the urban–rural category that defines the population density of the area in which the store was located.

Each sample was analyzed for a specific set of compounds, corresponding to pesticide residues that might occur in the fruit or vegetable from use of one or more organophosphate insecticides on the growing crop. Insecticides of interest are listed in Table 1.

Table 1 Parent organophosphates determined in OPMBS

Acephate	Malathion
Azinphos-methyl	Methamidophos
Chlorethoxyfos	Methidathion
Chlorpyrifos	Methyl parathion
Diazinon	Mevinphos
Dimethoate	Naled
Disulfoton	Oxydemeton-methyl
Ethion	Phorate
Ethoprop	Phosmet
Ethyl parathion	Terbufos
Fenamiphos	Tebupirimfos

This article summarizes key items discovered or developed during the OPMBS, to illustrate (a) the design of an LSMBS study protocol and (b) specific practices found useful in the conduct of such a study.

2 Development of study protocol

A number of important, interrelated items must be addressed in developing the protocol for an LSMBS, beginning with a clear articulation of the study objectives. Then a preliminary evaluation must be made to address fundamental concepts implicit in the study objectives, as follows:

- Is the issue of concern acute (short-term) or chronic (long term)?
- What products will be collected in evaluating the issue of concern?
- Do target populations exist, such as children, that require particular attention in the study design?
- Over what period of time should products be collected?
- From what types of sales outlets should products be collected?
- Will this be a national or regional survey?

- Will products be single-unit (e.g., individual piece of fruit) or composite (e.g., 5 lb of fruit to be blended together)?
- How many products will be collected (statistical sample size), i.e., will the tail of the distribution need to be defined, or will the mean sufficiently address the issue of concern?
- How much of each commodity will be collected (analytical sample size)?
- What type of measurement method may be employed?
- Which criteria are appropriate in examining the issue under investigation (e.g., method sensitivity)?
- How can products be practically packaged and shipped (e.g., can they be shipped in ambient temperatures or do they need to be shipped with ice packs or dry-ice)?
- How should results of the measurements be collected, compiled, and reported to best advantage?
- What lines of communication need to be established and enforced?

Detailed design can proceed when the above items have been considered and agreement has been reached on those important to the study. At this point, the roles and responsibilities of study personnel can be defined, development of commodity collection strategies can proceed, and consideration of measurement and reporting methods can begin.

In the development of the study design for an LSMBS, it is worthwhile to keep in mind that regulatory authorities have no established protocols or guidance for this type of study. If the results of the study will be submitted to a regulatory authority, it is advisable to meet with staff of the authority prior to finalization of the protocol, so that mutual understanding and consensus can be reached on the design.

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The OPMBS was designed by the Task Force's technical committee, in consultation with each of the three contributing contract organizations and the lead analytical laboratory. The overall design was predicated upon the objectives of the study. The major factor considered here is that the results of the study were intended to be submitted to the Environmental Protection Agency (EPA) to support dietary exposure and risk assessments. The results were also expected to be of interest to other agencies, specifically the United States Department of Agriculture (USDA), so both agencies (EPA and USDA) were consulted in the design phase of the study.

2.1 Definition of study objectives

The design of an LSMBS must proceed from a defined study objective. The study objective is the basis for all decisions made in the design and conduct of an LSMBS.

One common objective of an LSMBS is to refine the estimates of actual exposure of consumers to ingredients or impurities in one or more products. For example, study results might be intended to determine a realistic human dietary exposure to pesticide residues in fresh fruits and vegetables. The advent of the Food Quality Protection Act of 1996 (FQPA) has produced an enhanced focus on the exposure of children to pesticides. A well-designed and implemented LSMBS would afford the opportunity to delineate better the exposure and risk to children and other population subgroups. The LSMBS would provide consumer-level data at or near the point of consumption, allowing the refined, relevant, and realistic assessments of dietary exposure.

The OPMBS was typical of an LSMBS, and began with a definition of the study objectives. The primary objective of the OPMBS was to determine the frequency of occurrence and the magnitude of particular pesticide residues in/on foods that people actually consume. This objective is distinctly different from that of typical field residue studies conducted to support pesticide registrations. Field residue studies determine the concentration of the pesticide immediately after the commodity is harvested. A market basket survey, however, is concerned with commodities at the consumer level, i.e., obtained from commercial retail outlets rather than from controlled test plots. This distinction is important, because at harvest, commodities from regulatory field residue studies generally contain the maximum levels of residues, as one outcome of the worst-case testing regime required by the EPA (highest rates of application, shortest interval between application and harvest, and analysis immediately thereafter). Commodities actually purchased and used by consumers, however, almost always contain lower levels of residues, because (a) more realistic application and harvest regimes are used, (b) residue levels can diminish between harvest and sale, and (c) not every commodity will have been treated with a given pesticide.

2.2 Role and responsibilities of study personnel

To be successful, an LSMBS requires a clear definition of the responsibilities of each participating individual or group. Preparation of an organization chart may be appropriate, as would its inclusion in the study protocol. Key study participants could include Study Directors, Principal Investigators in the sample collection and analytical phases, sponsor representatives, technical consultants, residue analytical laboratories, and QA specialists.

The OPMBS was organized as follows. The overall responsibility for the study was held by a seven-person technical committee, populated with a representative from each of the sponsoring agrochemical registrants. Reporting to the technical committee were three individuals: the Study Director, the collection coordinator, and the study QA specialist. The Study Director had responsibility for implementation of all technical aspects of the study and served as the main contact with the four residue laboratories involved. The collection coordinator had responsibility for statistical design of commodity sample collection and for actual collection and shipment of the samples to the laboratories. The collection coordinator also served as the main contact with the sample collectors, i.e., shoppers. The study QA specialist had responsibility for verifying adherence of the study to relevant Good Laboratory Practice (GLP) and other standards. The study QA specialist worked with QA staff at the different facilities, with shoppers, with the collection contractor, and in certification of the final study report.

All individuals involved in the technical aspects of a study must have a clear understanding of the objectives and special constraints of the study. A common understanding is particularly important in an LSMBS, because several hundred individuals are typically involved in such a study. Consistency among the groups performing similar activities is crucial to the outcome of the study and must be a primary consideration.

The OPMBS featured a 2-day organizational meeting between study management, including the Study Director and sample collection coordinator, and representatives

of each of the four residue laboratories. Several members of the task force technical committee also participated. The meeting, held about 3 months prior to the start of collection of commodities, had several purposes. First, each of the representatives was able to meet his or her counterparts in other (normally competitive) laboratories on neutral territory. Second, each representative received the same description of the study and its objectives and its organization. Third, each received the same information on how the analyses were to be carried out. Fourth, and probably most important, each received a description of how the results were to be assembled and reported. At the time, these items were at the proposal stage, and each representative had the opportunity to comment on the proposal and offer alternatives. Many of the items raised by the laboratory representatives, in fact, were incorporated into the final plan.

The organizational meeting had several positive outcomes, which could have been reached in other ways only with difficulty. First, everyone involved realized that once the data collection and reporting process had been agreed, each laboratory would be expected to follow it, without modification. This realization was possible because the procedures to be used after the completion of the analyses were clearly explained, and the need for consistency in reporting was abundantly clear. Second, it was possible to reinforce the lines of communication and responsibility that everyone involved in the study was expected to observe. Third, the necessity for rapid communication of problems between study management and field and laboratory personnel was underscored.

2.3 Selection of products and of properties to be evaluated

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In general, an LSMBS is intended to address the properties of products at the consumer level. In the discussion below, the property is pesticide residues, and the product is commercially available fresh fruits and vegetables. However, similar considerations apply to the selection of other types of properties in other products, such as ingredients in hand lotions.

A typical field residue study has little or no latitude in the selection of commodities to be analyzed. Guidelines specify the commodities that must be analyzed for most crops, and for any crop that lacks specific guidelines, commodities can be selected by inference. For an LSMBS, in contrast, the commodities can be chosen from the universe of food items available to consumers. The final selection of commodities might consider such factors as (a) the portion of the consumer diet represented by the commodity, (b) whether the commodity makes up an appreciable part of the diet of a subcategory of consumers, such as children, and (c) if the commodity is likely to be available throughout the geographical coverage area and the study period. For example, a 1-year study considering the dietary burden caused by treatment of blueberries with a fungicide may not be feasible because of the highly seasonal nature of blueberry culture.

Because the conclusions that can be reached from an LSMBS depend critically on the choice of commodities, involvement of relevant regulatory agencies [e.g., EPA, USDA, and Food and Drug Administration (FDA)] in the USA, depending on the commodity and study objective] should be considered. Agreement on the choice of commodities by such authorities will ensure that the study and its outcome will be acceptable for regulatory purposes.

Nearly any group or type of commodity can be included in an LSMBS, provided that sufficient thought is given a priori to the definition of the commodity and the information the investigators intend to generate. Thus, an LSMBS might be designed to determine residues of organochlorine insecticides in milk products in a particular production period within a certain area within the southern USA. Other LSMBSs might focus on processed food items, or animal products, or eggs.

Pesticide residues consist of chemicals that might occur in a commodity as a result of application of a pesticide. Such chemicals typically correspond to compounds for which a regulatory agency has or will set a tolerance, i.e., a maximum residue limit, specific to the commodity. In either a field study or a market basket survey, residues to be determined will be those which result from application of the specific pesticide that the study is intended to support. A market basket survey, however, might be intended to support not just one but several different pesticides of the same or different chemical classes. In addition, a market basket survey might include pesticides not used in the USA but for which import tolerances exist. For example, some uses of the parathion family of pesticides on food products have been abandoned in the USA but remain in other countries that export the products to the USA. A market basket survey offers a means to evaluate actual dietary exposures to residues of such pesticides. In addition, tolerance expressions frequently include multiple compounds, all of which must typically be determined in residue field trials. The sponsor of the market basket survey must decide whether to analyze for all compounds in the applicable tolerance expression or to restrict the program to selected analytes, such as the active ingredient.

In order to select the fresh fruits and vegetables for the OPMBS, each task force member company provided a list of commodities that the company considered to be the most important for its organophosphate products. A matrix was developed based upon a crop group strategy and the importance of commodities in the diet of infants and children. Thirteen fresh fruits and vegetables were eventually selected for inclusion in this study.

In the OPMBS, all compounds were determined that occurred in the tolerance expression for organophosphate pesticides being supported by the registrants in each of the 13 commodities. Thus, compounds to be determined were those that might occur as residues of organophosphate pesticides whose continued use was supported by members of the OPMBS Task Force.

2.4 Sample collection strategy

The protocol must include in detail the strategy for collection of the chosen commodities, i.e., the sampling plan. All aspects of the sampling plan must be consistent with the study objectives, including the number of individual commodities to be collected, the geographical area from which they will be drawn, the type of stores from which commodities will be collected, and the frequency and period over which collection will be done. Therefore, the sampling plan has to be well thought out, again beginning with clear statements of (a) the study objectives and (b) how the study results will satisfy the study objectives.

Because the sampling plan is central to the overall objective of an LSMBS, the plan should be statistically designed. The overall intent of the design is that

the commodities collected represent the contribution of the commodity to the diet of the targeted consumer population. In essence, the sampling plan takes the study design from the general, i.e., what commodities, what geographical distribution, what type of outlet, and what time-frame, to the specific, i.e., how many individual samples of each commodity will be obtained, from which specific stores in which locations, and on what dates.

Restrictions, such as avoiding collection of organic or hydroponically grown items, should also be considered. Details such as how many samples of each commodity must be obtained on each sampling date, minimum sample weights, and precise commodity definition, i.e., head versus leaf lettuce, must be included in the plan. Furthermore, the sampling plan must allow for contingencies. Thus, the plan should specify what action the shopper should take if, for example, (a) a commodity is not available at the prescribed source, or (b) circumstances prevent shopping on the prescribed day.

The target number of commodity samples to be obtained in the OPMBS was 500, as determined using statistical techniques. A sample size of 500 provided at least 95% confidence that the 99th percentile of the population of residues was less than the maximum residue value observed in the survey. In other words, a sample size of 500 was necessary to estimate the upper limit of the 95% confidence interval around the 99th percentile of the population of residues.

Once the target number of samples was defined, the frequency of collection and the number of samples to be collected on each collection date were determined, based on an overall total sampling period of 1 year. The sampling plan specified collection every other week, primarily to accommodate the workload at the analytical laboratories. Sampling had to occur early in the week to preclude problems with shipping samples over the weekend. With these considerations in place, specific dates for collection of commodity samples could readily be set.

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In the OPMBS, a statistical design for the selection of stores from which samples could be collected was developed. This design used key factors including the geographic location of the store, the size of the store, and the population density of the area in which the store was located. In addition, secondary stores were designated. Secondary stores were used when a commodity was not available at the primary store, or the primary store was not accessible. Primary and secondary stores were chosen so that the demographics of the selected stores reflected the overall population.

The sampling plan for the OPMBS required that no organic or hydroponically grown produce be obtained, that the precise commodity, i.e., head, not leaf lettuce, be collected, and that only two representative single-serving samples of each commodity be collected on a given date by each of 20 shoppers. The plan also specified two back-up (secondary) stores for each primary store and instructed that the collection coordinator be notified if samples could not be collected on the specified date so that the sample collection could be rescheduled.

2.5 *Analyses and data reporting*

An LSMBS typically involves determination of one or more properties in a product or group of products. The following discussion is specific to the determination of residues in foods, but the considerations also apply to other properties in other products.

Residue study protocols typically either include quality specifications for analytical procedures or refer to a written analytical method that includes such specifications. The protocol for an LSMBS should also include analytical quality specifications, either directly or by reference to a method. Analytical specifications usually include minimum and maximum recovery of analyte from fortified control samples, minimum number of such fortifications per set of samples, minimum linearity in calibration, minimum stability of response to injection of calibration solutions, and limits of quantitation and of detection.

For an LSMBS, consideration must be given to the constraints of analytical methodology in setting quality specifications. For example, achieving recovery from fortified controls can be difficult when multiple analytes, with different physical and chemical characteristics, must be determined. The difficulty arises because optimal analytical parameters for one analyte may be very different to optimal conditions for another. Thus, setting recovery limits too narrow might result in rejection of results and extensive reanalyses, when the objectives of the study could readily be achieved with a wider range of allowable recovery.

As discussed above, field residue studies and market basket studies have different objectives. The purpose of a field residue study is to set tolerances. In contrast, the purpose of the OPMBS was to screen a large number of samples of a large number of commodities for a large number of analytes. Thus, in developing the protocol for the OPMBS, the technical committee determined that the purpose of the study allowed a wider range of recovery than is typically specified for field residue studies.

Similar considerations were taken into account throughout the process of designing the study and committing the design to a protocol. In addition to analytical quality specifications, decisions were made regarding definitions of limits of detection and quantitation, levels of apparent residues at which confirmation was required, and how such confirmation would be achieved. All of these decisions were based on fulfilling the objectives of the study while operating within unavoidable time and resource constraints.

3 Implementation of sampling plan

3.1 Shopper selection and training

Implementation of an LSMBS sampling plan requires consideration of logistics, beginning with personnel. The plan generally will require the collection of products from a wide geographical area, so a large number of individual shoppers must be identified and trained. Ideally, shoppers will have some degree of familiarity with products to be collected. For example, shoppers for a food-based LSMBS are typically registered dieticians and are located through professional affiliations. Some consulting firms maintain extensive listings of individuals who serve as shoppers in particular geographical areas.

Each shopper must be provided with clear, concise oral and written directions, e.g., where to shop, what and how much to collect, how to package and label the samples, and where and how to ship the samples. Information must be provided on the exact products to be purchased, including any limitations. Some of the limitations in a

food-based LSMBS might include collection of only certain varieties, of non-organically grown produce, of produce not identified as imported from outside the United States, of produce without blemishes, etc. The directions provided to the shoppers should reflect the overall study design.

The training of LSMBS shoppers is generally conducted by telephone and through written instructions. The written instructions and forms provided to the shoppers form the equivalent of a standard operating procedure (SOP). Often, since the network of shoppers is widely dispersed and may or may not be involved with more than one sample collection period, formal SOPs are not devised and provided to the shoppers. Instead, complete written instructions are provided, albeit not necessarily in SOP format.

In the OPMBS, shoppers were selected from a list maintained by the sample collection coordinator and were for the most part trained dietitians. Each shopper received at least three in-depth telephone contacts prior to sample collection. Each shopper also received written instructions.

3.2 Sample collection, storage, shipment, receipt, and documentation

The field phase of an LSMBS is critically important. Close monitoring of shoppers by field phase study management personnel is required, especially when a sampling plan includes frequent collections, such as weekly or bimonthly. Missed, delayed, or deficient commodity samples can throw a laboratory off schedule, which in turn can adversely affect both the timeliness and the quality of the analyses. Missed, delayed, or deficient samples can also affect the study outcome and interpretation, because a statistical design typically requires a certain number of data points, each represented by analysis of a commodity sample.

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Communication among all individuals involved in an LSMBS is crucial. Lines of communication must be clearly defined and must be in place well before the study is initiated. Therefore, lines of communication between the persons who collect and ship commodities, the field phase management study personnel, the analytical laboratories, and overall study management must all be clearly established prior to study initiation. This delineation of the lines of communication must include oral and written communication, including the transmittal of raw data such as sample collection logs and chain of custody forms.

Sample commodities may be stored by the shopper prior to shipping, and storage should reflect typical consumer practices. For example, if the commodity is a potato, then the shopper need not refrigerate the sample prior to packing it for shipment. If, however, the commodity is perishable, such as milk, then the sample should be refrigerated prior to packaging for shipment. The way in which the samples are to be packaged and shipped must be defined in the shopper's instructions, as discussed above. Fresh produce should be shipped with ice packs to ensure its freshness upon arrival at the analytical laboratory. Nonperishable foods such as canned goods, however, can be shipped under ambient conditions.

Upon receipt at the analytical laboratory, the receiving department should immediately examine the integrity of the samples. If a sample is damaged, or its integrity is in any way questionable, or it does not meet the protocol definition (e.g., leaf rather than head lettuce), then a re-shop should be ordered (i.e., the shopper is required

to go back to the store and collect a second exemplar of the commodity). Written documentation in terms of the chain of custody forms, and also additional records by the field phase management study personnel and analytical laboratory personnel, must be maintained to reflect the storage and shipping status of the samples.

Adequate pre-shop provision, thorough training, and strict oversight of the shoppers, as described above, were critical to the successful execution of the sample collection phase of the OPMBS. Each shopper received a kit containing sample labels and containers to hold the sampled commodities, ice packs and packaging materials, labels and boxes for use in shipping the collected commodities, written instructions, and forms well before the scheduled date of collection. The sample coordinator monitored sample collection and advised shoppers of actions to take when problems inevitably arose.

Contact with the shoppers was restricted to field phase management study personnel, for two reasons. First, clearly defined lines of communication had to be maintained. Second, in order to ensure that the identity of the stores remained blind (i.e., unknown to everyone downstream from sample collection), in compliance with one of the design criteria, communication with the shoppers had to be restricted. Overall, limiting contact with shoppers to one entity and using modern technology, such as facsimiles and e-mail to facilitate and document communications between shoppers and the collection coordinator, were essential factors in the successful conduct of the sampling phase of the study.

Each analytical laboratory inspected shipments as they were received, and documented the receipt of the samples and their condition. Any problems were immediately communicated to the appropriate field phase management study personnel to facilitate immediate corrections, such as re-shops required because the wrong commodity had been sampled.

4 Analytical phase

4.1 Analytical method

In any residue program, but particularly in an LSMBS, a well-established and validated method should be used. The alternative is to carry out method development and validation in parallel with the study, a course of action that is not recommended.

Because an LSMBS is almost certain to involve more than one laboratory in the analytical phase, results obtained by multiple laboratories must be internally and externally consistent. For this reason, the use of a single method in all analyses, if possible, is advantageous. The method must conform to quality criteria and must be rugged, i.e., must be satisfactory for all analytes in all commodities, with instruments and data acquisition systems from various manufacturers.

In the OPMBS, the lead laboratory developed the analytical method for all analytes in all commodities. The same laboratory validated the method for each commodity, to demonstrate that all the specific analytes for the commodity could be determined in accordance with analytical quality specifications. The method was then provided to the other three laboratories, each of which validated the method for its assigned commodities, to ensure that the method performed properly using the laboratory's equipment and personnel.

4.2 Obtaining control commodities

As with any residue method, a method used in an LSMBS method should include analysis of control commodities to demonstrate adequate selectivity and analysis of fortified control samples to demonstrate recovery. These aspects present a particular challenge in every food-based market basket survey, because, unlike field residue studies, control samples of known provenance are not available.

In a field residue study, commodities are grown on control plots located near the plots used to produce commodities treated with test substance. Care is taken to ensure that the only difference between control and treated commodities is that the former does not receive application of the pesticide and the latter does. Crop variety and growing conditions (including geographical location, soil, time of year, weather, etc.) are essentially identical for the control and the treated commodities.

A market basket survey, however, is unique in that untreated control commodities, as the term is normally used in residue studies, cannot be obtained. In a market basket survey, food commodities are collected at the consumer level and not from controlled field tests. By design, the cultural and treatment details for the collected commodities are expected to differ from sample to sample. This factor enables the collected commodities to represent the spectrum of conditions under which crops are supplied for human consumption.

The use of controls is nevertheless necessary, as stated above. Organically grown commodities can be used as controls, subject to prequalification to ensure the absence of interference. In the OPMBS, candidate control commodities were purchased from markets specializing in organic (pesticide-free) produce or from individual growers. Each candidate control commodity was qualified by analysis for the suite of analytes to be determined in actual samples of the commodity. This strategy worked well for the most part, with only a few difficulties. First, organically grown exemplars of some commodities were not locally available at all times. This problem was exacerbated by seasonality but was solved by purchase in other geographical areas. For instance, organically grown peaches and green beans from suppliers in California were purchased and shipped to laboratories in the Midwest. As a second example, organically grown peaches were not available anywhere in the United States in winter, so control commodities were obtained from the Southern Hemisphere.

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A second problem was that some lots of control commodities contained one or more extractable interferences, i.e., co-extractives that interfered with one or more of the analytes for the particular commodity and could not be removed during cleanup. This problem was addressed by either using controls from different sources for specific analytes or by blending controls to obtain a matrix with a sufficiently low level of interference to allow accurate determination of recovery.

4.3 Assignment of products to laboratories

Typically, an LSMBS will require more analyses than can reasonably be conducted by a single laboratory. Therefore, the analytical workload will have to be divided, with at least two and possibly more laboratories participating. Analytical results are generally more reliable and consistent if the individuals performing the analyses

have experience with how the method works on the specific product to be analyzed. Therefore, assigning all analyses of any one matrix to a single analytical laboratory is preferable to shifting the analyses among laboratories.

In deciding which laboratory should analyze which products, both the relative difficulty of analysis of each matrix and the capacities and capabilities of the laboratories should be considered. Difficult matrices could be divided among the laboratories or assigned to the laboratory that is considered most capable of dealing with the difficulties. Alternatively, the matrices might be divided by type, so that in a food-based LSMBS, for example, one laboratory might analyze only fruit, another only leafy vegetables and brassica, and a third only root and tuber crops, so that each laboratory would face only one set of problems in analyzing only one type of matrix.

Commodities in the OPMBS were assigned to laboratories based on the precept that no one laboratory should be overburdened. Thus, the most difficult commodities, based on information obtained during method development, were distributed among all four participating laboratories.

4.4 Standardization of results reporting

The objective of an LSMBS is not simply to collect and analyze samples of selected products. To be of value, the results of the analyses must be reported, and standardization in reporting of intermediate and final results is critical to the success of the overall project. Each laboratory should determine exactly the same analytical parameters, calculate results in exactly the same way, and present both inputs to and outcomes of the calculations in exactly the same format.

Imposition of this requirement has a number of immediate benefits. First, inconsistencies among the laboratories in recording data and in calculating and reporting results are all abolished. Second, the imposed consistency greatly simplifies review of the findings, both internally by study personnel, QA, and management and externally by regulatory agencies. Third, little, and ideally no, manipulation of results is needed in preparing the final report. Besides facilitating the reporting process, data re-entry or copying can be entirely avoided, which also minimizes the amount of review needed in QA. Finally, a consistent format that includes presentation of quality assurance parameters such as recovery from fortified control, linearity of response, etc., ensures that all laboratories are subject to and meet identical quality assurance specifications.

The OPMBS used a custom-written spreadsheet application, i.e., a workbook, in conjunction with laboratory automation systems to standardize data recording, calculations, and presentation of results. Devising this approach required careful differentiation between (a) the workbook used to calculate and report the results and (b) the data acquisition systems used in each laboratory. The laboratory systems were used to collect the raw chromatographic data, but the calculation modules in the laboratory systems were not used. Instead, all calculations were done in the workbook. Use of the laboratory systems to collate and output the final results was considered but was rejected for two reasons. First, different laboratories used different systems, and some laboratories used more than one system. The output characteristics of the various systems differed considerably and would have required extensive modification

to produce output that contained the same information in the same format. Second, the manufacturers of some of the systems do not provide details of calculations, so, in principle, reconstruction of reported results could have been difficult. These issues were obviated by use of the workbook.

Each commodity required a specifically customized workbook, containing a worksheet for each analyte determined in the commodity. Each laboratory received electronic copies of either three or four workbooks, which served as templates for the three or four commodities assigned to the laboratory. Each set of up to 10 commodity samples scheduled for collection and analysis required the creation of a copy of the appropriate template. Each workbook template contained one primary worksheet for each analyte, in which analytical data were recorded and residue levels were calculated, as described below. For example, the template for green beans contained 17 primary worksheets, one for each of the 17 analytes determined in each green bean sample. Additional worksheets were inserted into copies of the template as needed, to describe results of further analyses, such as confirmation of analytes present above the limit of quantitation (LOQ) or dilutions to bring the concentration of the analyte into the calibration range.

In addition, each workbook contained a summary table of all results and limit of detection (LOD) determinations. The table was organized with sample identifications in the left-hand column. For each analyte, the analytical result and the LOD appeared in adjacent columns, and analyte recoveries appeared above the results columns. The summary table was generated automatically from the analytical results in the individual worksheets, without operator intervention or re-entry of any information.

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Information from the summary table in each workbook was directly imported into a master compilation of analytical results. The compilation could then be manipulated as desired to present the data in various ways. For example, the compilation could be searched for the number of apple samples that contained no detectable residues of any analyte or for the number of tomato samples that required dilution and reanalysis.

The OPMBS workbooks required the user to enter sample identification and the dates of collection, extraction, and analysis. Additionally, entries were required for the analytical responses of calibrants, the corresponding responses of sample extracts, and the parameters needed to calculate the LOD. Given the required input information, the spreadsheet automatically calculated and displayed, for each analyte:

- the interval between sample collection and extraction
- the calibration curve (displayed graphically) and its goodness-of-fit
- a back-calculation of analyte concentrations from responses to injections of calibrants
- the recovery of analyte (tested in every analytical set)
- the residue if any found for each sample
- an indication of whether the range of calibration was exceeded and
- the LOD.

Each individual spreadsheet contained several logical tests ensuring that no calculations or results could be reported unless all members of a required raw data set were entered. Control over the use of the spreadsheets was provided by issuing the workbook templates to the laboratories in forms that contained locked and unlocked

cells. The unlocked cells were those into which data was required to be entered. As described above, omission of data from any of the necessary cells precluded completion of the calculations and avoided the submission of partial results. The locked cells contained, primarily, the algorithms, logical tests, and QA criteria established a priori by study management.

The locked cells in any worksheet could not be opened or altered by laboratory staff without the knowledge and agreement of the Study Director. Use of the locking function in the workbook, therefore, constrained the analytical laboratories to a single system for recording data and calculating results.

When a notebook had been completed, and the external and QA reviews had been finalized, the laboratory coordinator locked the entire workbook, using a macro specifically developed for the purpose, and sent the workbook for collation into the final study report. Once locked, the workbook could not be altered without the knowledge and agreement of the laboratory staff responsible for generating and certifying its contents. Such a system is critical in order to maintain integrity of reported results.

4.5 Presentation and review of study findings

In any study involving analyses, part of the responsibility of management at the analytical laboratory is the review and approval of intermediate and final reported results. In an LSMBS, such review and approval must take place at each analytical laboratory involved in the study. However, different laboratories may focus on different aspects of the analyses, and some means to ensure that review procedures and approaches are consistent among the laboratories is needed. It is advisable, therefore, to include an additional review, termed here an 'external review', beyond that conducted by the individual laboratories.

External review is of major importance in ensuring the outcome and reportability of LSMBS study results. Additional experts have the opportunity to review the data and results just after their generation, at a point where corrections can be easily proposed and made. In addition, external review aids in achieving consistency in the results reported by different laboratories. Finally, external review provides feedback for optimization of the analytical and instrumental parameters at each laboratory.

Each laboratory in the OPMBS was required to send a copy of each workbook and the associated raw data, to study management soon after completion of the analysis. This information was provided after internal technical review but before local quality assurance review. Study management used a team of experienced residue chemists to review the results and the raw data and to ensure that the information reported was fully supportable. The laboratory remained at all times responsible for the data reported, however, and the results of the external review were formally considered to be advisory.

The external review pointed up several types of occurrences that had to be addressed at the laboratories. In some cases, corrections had to be made, for example if elution peaks had been misidentified or if the laboratory data system integrated noise and reported a peak where there was none. Such occurrences were observed primarily in the early stages of the study and decreased as the laboratories refined integration parameters for their individual commodities. In other cases, it became apparent that

certain operations were being carried out or reported differently among the various laboratories, and consistency had to be ensured. For example, LOD values calculated by one laboratory were unreasonably low at the start of the study, and the method that the laboratory used to derive a value for baseline noise had to be modified.

In the OPMBS, external review was conducted exclusively by one individual during the early months of the study. This practice achieved consistency of reviews among the laboratories. Furthermore, finding a problem during review of raw data from a given laboratory frequently triggered closer examination of data from other laboratories. A number of issues were thus resolved quickly and consistently in the early part of the study. After the initial months of the study, experience allowed the categorization of the types of problems likely to emerge, and additional reviewers were added to the program.

5 Quality assurance functions

QA is an important aspect of any technical study. It is particularly crucial in an LSMBS, because several hundred participants, widely separated geographically, are involved. The analytical laboratories typically have standard provisions for QA inspections and reviews, and the field phase management organization is also likely to have standard provisions for QA inspection and review. Shoppers, however, are typically external to study management and analytical laboratories and, thus, are not directly covered by existing QA systems. The study design must include a means by which the field phase, i.e., sample collection and shipment by the shoppers, is made to comply with QA requirements.

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Each contract laboratory used in the OPMBS and the organization that coordinated the field phase already had in place a well-defined GLP-compliant quality assurance program. In addition to these existing quality assurance programs, an independent QA specialist was engaged to ensure compliance of all the aspects of the study with GLP requirements.

The study QA specialist visited each laboratory, conferred with the local QA specialist, and reviewed local practices and record keeping, thereby establishing that personnel and practices at each laboratory were satisfactory. Each laboratory, of course, remained responsible for internal compliance with the protocol and with GLP requirements throughout the study.

Thereafter, the primary functions of the study QA specialist fell into two main headings. First, GLP compliance during the collection and documentation of commodity samples had to be assured. This was done via observation of several collections for different shops (collection incidents) at various geographic locations. In these audits, the study QA specialist examined specific items, such as (1) did the shopper follow the written instructions, (2) were the correct types and numbers of samples collected, (3) was the documentation maintained as required, (4) were the samples labeled and packaged correctly, and (5) were the samples delivered to the shipper as required? Findings were communicated to study management and used as appropriate in subsequent shops.

The second main function of the study QA specialist was to review and validate the assembly of the final study report. As developed above, the workbooks containing the

analytical results underwent QA review at the generating laboratories and could not be modified thereafter. Steps carried out during preparation of the final report, however, required that the results be read into a large database and then sorted and manipulated mathematically and statistically. Additional QA steps were taken to ensure the validity of summaries and statistical treatments prepared using the results in the database.

6 Interpretation of study findings

LSMBS findings can be interpreted and used in a variety of ways. The overall data set can be examined by evaluating trends as well as statistical evaluation. For example, trend analysis in a food-based LSMBS could include determining if there are any particular residue patterns for commodities within a season, region, or type of analyte. Statistical evaluation may include the determination of statistical parameters such as mean, minimum, maximum, median, and any given percentile. Data may also be compiled to determine the number and percentage of samples with quantifiable or nonquantifiable residues. The manipulations can then be used in interpreting the data and also for regulatory and/or business decision making. The data may also be used in refined risk assessments, such as those required by the FQPA. Another use may be to develop translation factors between, for example, residue field trial data and consumer (i.e., LSMBS) data. Such translation factors can then be applied more broadly than the empirical data, itself, supports. Direct and indirect (i.e., through the use of translation factors) use of LSMBS data affords many opportunities for utilizing these data-rich, robust data sets.

OPMBS data were intended to support a valid estimate of the dietary exposure of populations and sub-populations to organophosphate residues in fresh fruits and vegetables. The results of the study were presented to the EPA in a report, with appropriate summaries. All of the study results, i.e., residue levels of each compound determined in each sample of each commodity, were also provided to the EPA in a database. EPA has recently notified the task force that the OPMBS study on the frequency and magnitude of organophosphate residues in fruits and vegetables is acceptable. The EPA is expected to utilize the data in a new assessment of potential dietary risk from organophosphate residues.

Sampling and analyses of foodstuffs from animal origin

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1 Introduction

Prior to regulatory approval of animal drugs or pesticides, a suitable analytical method for monitoring violative residues in foodstuffs of animal origin (meat, milk, and eggs) is necessary. For animal health drugs in the USA, legal authority for requiring analytical methods is established under the general safety provisions of the US Food Drug and Cosmetic Act[1] as promulgated in the Code of Federal Regulations.[2] The Center for Veterinary Medicine (CVM) of the Food and Drug Administration (FDA) has responsibility for evaluating these methods prior to approval. For pesticides in the USA, the Environmental Protection Agency (EPA) has responsibility for evaluation of the analytical methods used for monitoring pesticide residues in foodstuffs of animal origin. The Food Safety Inspection Service (FSIS) of the United States Department of Agriculture (USDA) has responsibility for using appropriate analytical methodology to monitor foodstuffs of animal origin for violative residues of both animal drugs and pesticides as part of its National Residue Program.[3] In Europe, the authority for approval of analytical methods in foodstuffs is given to the Committee for Veterinary Medicinal Products (CVMP) of the European Agency for the Evaluation of Medicinal Products (EMEA). A European Directive[4] has called for transfer of surveillance analytical methods to a Community Reference Laboratory (CRL) or National Reference Laboratory (NRL) in each Member State of the European Union (EU), which have the responsibility for monitoring foodstuffs of animal origin for violative residues. In both the USA and Europe, regulatory limits of residues in edible tissues are established for approved drugs and pesticides, which are used to monitor for appropriate use of those drugs. For drugs in the USA, this concentration limit is called a 'tolerance', and in Europe and the rest of the world the concentration limit is called a 'maximum residue limit' (MRL). These limits are defined in terms of a 'marker residue', which may be the parent drug or a major metabolite in a specific tissue or tissues. Considerable residue data are necessary to establish regulatory limits,[5,6] but a discussion of the establishment of these regulatory limits is outside the scope of this article. Two methods are necessary in the USA and Europe for regulatory approval: a determinative

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method providing for a quantitative determination of the residue concentration and a confirmatory method providing for structural verification of the residue. The purpose of this article is to discuss strategies that will lead to the successful development and validation of determinative and confirmatory analytical methods.

Although requirements necessary for the approval of determinative or confirmatory methods differ depending upon the regulatory agency (see Section 6 for a discussion of validation guidelines from different regulatory agencies), the general principles necessary to create a successful method remain the same and include precision, accuracy, sensitivity, selectivity, ruggedness, and practicability. Precision and accuracy are important variables to ensure that a measurement is not too variable or does not have systematic errors. The sensitivity of an analytical method is critical to ensuring that the method has adequate performance at and below the level of the tolerance or MRL. Selectivity or specificity must be considered with respect to interferences that may occur due to the tissue matrix, metabolites, and other drugs that might also have been used. Ruggedness is critical because the methods developed will be used not just in the developing laboratory but also in a number of other laboratories and potentially over a long period of time. Practicability is a concept akin to ruggedness that ensures that the method does not have steps that would be unfamiliar to a reasonably experienced analyst or use uncommon reagents or equipment. All of these parameters must be considered prior to development of a tissue residue method, otherwise considerable investment of time can be wasted on a method that will ultimately be unsuccessful when transferred to other laboratories.

Another equally important consideration before development of a determinative or confirmatory method is an understanding of the chemical properties of the analyte. Such an understanding becomes the cornerstone of a successful method since the unique chemical properties of each analyte provide the basis for isolation and detection schemes. Table 1 lists some of the important chemical properties that could be considered. For example, knowing the pK_a or pK_b of an analyte could influence the choice of a liquid–liquid extraction scheme, solid-phase extraction (SPE) cartridge, mobile phase pH, or mass spectrometric ionization. Knowing the overall polarity of the analyte can be very helpful in the evaluation of an extraction or separation. Currently, computational methods[7] are available to obtain an estimate of the $\log P$

Table 1 Chemical properties important in method development

Physical property	Measure
Acidic or basic functionality	pK_a or pK_b
Polarity	$\log P$
Vapor pressure	Boiling point
Molecular weight	Atomic mass units
UV absorbance	λ_{max}
Fluorescence	Minimum excitation and emission wavelength
Electrochemistry	Oxidation or reduction potential
Chirality	Enantiomers and diastereomers
Solubility	mg compound dissolved in mL of solvent
Stability	Loss with time (%) under different storage conditions
Special atoms	Cl, Br, P, and S
Special functional groups	Primary, secondary, and tertiary amines, carboxylic acids, alcohols

value (octanol–water partition coefficient) without experimental determination. For gas chromatography (GC), the presence of a halogen, sulfur, or phosphorus atom may influence the choice of a detector. The presence of a chromophore [ultraviolet (UV)/visible], fluorophore, or electrochemically active group may lead to the selection of the liquid chromatographic detector. Another important consideration is the potential negative consequence of a certain functional group. For example, amines and carboxylic acid groups are notorious for secondary interactions with reversed-phase stationary phases in liquid chromatography (LC). Measures to counteract this deleterious effect may be necessary. Further, a combination of properties can complicate the overall decision-making process. For example, zwitterions may contain both amino and carboxylic acid functionalities such that the molecule has functional groups that are charged at any pH. Another related example is the dramatic change in the polarity that occurs on changing the pH of the solution for molecules that have an acidic or basic functional group. Taken together, assessment of all of these properties from the very beginning is critical in ensuring that the method has the best chance of success.

The subsequent sections will provide a summary of the literature with respect to determinative and confirmatory methods developed (Sections 2–5) and validated (Section 6) for animal tissues (meat, liver, kidney, fat), milk, and eggs. Although the method development sections are organized around the analytical techniques used in current methods, special emphasis will be given to characteristics of the analyte that make this technique the best choice. Also, this review will be retrospective, pulling relevant examples from the literature, but the guidance provided will be prospective looking at best practices for future methods. Therefore, techniques such as microbiological assays will not be discussed because they have been largely supplanted by other techniques. Finally, regulatory requirements necessary for a successful determinative or confirmatory method will be considered throughout even though Section 6 will provide a detailed discussion of regulatory method validation parameters.

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2 Sample collection and storage

2.1 Sampling and homogenization

Collection of tissue, milk, or egg samples is an important first step in the method development process and must be addressed with specific procedural steps in the analytical method or sample collection standard operating procedure. Whether the method is for tissue, milk, or eggs, the samples must be collected in such a manner as to minimize the possibility of cross-contamination. Tissue samples are collected at the time of euthanasia and exsanguination. To minimize the risk of sample contamination, a sharp knife or scalpel should be used to isolate the specific tissue of interest from extraneous tissue material, and copious water should be used to wash away any blood or fecal matter that may remain. Further, the tissue sample should be collected in a large enough sample size to ensure homogeneity. For large animals, sub-sampling the liver is appropriate, but samples must be taken from each distal lobe and be of significant size to reflect adequately the whole lobe (i.e., 1 kg from each distal lobe). Poultry livers are generally collected in their entirety. Kidneys are generally collected without sub-sampling, but care should be taken to remove any renal

fat. For muscle, sub-sampling from more than one muscle location may be necessary. While most drugs appear to distribute equally into muscle tissues, several studies[8–11] have shown that drug residues in muscle tissue can be distributed differently depending upon muscle type. Therefore, muscle tissue samples may require collection of sub-samples from various muscle groups such as shoulder and loin muscle in cattle or white and dark muscle in poultry. Fat (and skin with attached fat for poultry) is similar to muscle in that sub-sampling in such a way as to be representative of fat in various locations within the animal may be required. For example, pyrethroids are distributed differently to abdominal fat and renal fat, so in Australia renal fat is evaluated separately. Milk samples can be collected from a bulk tank or individual cows. Care should be taken to ensure that the milk has not separated prior to sampling. For samples collected from individual animals, milking equipment needs to be appropriately cleaned to prevent cross-contamination from occurring with previously collected milk. Egg sample collection is the most straightforward as each egg is an individual sample unit.

Generally, samples are collected at the site of necropsy and transported to a laboratory for homogenization. Since time can elapse between sample collection and homogenization, samples must be stored in a manner appropriate to ensure sample stability (for example, samples are frozen within a certain time after their collection). Because the entire sample will rarely be consumed in a single analytical assay, the sample almost always needs to be homogenized and then sub-divided into an appropriate size for further assays. Also, tissue homogenization procedures usually reduce the sample into smaller particle sizes, which increases the surface area and aids in extraction efficiency. Several homogenization techniques have been described in the literature but generally involve grinding, chopping, or mixing. For small tissue samples such as poultry liver and kidney, a sharp scalpel can be used to mince and blend the tissue into a uniform mass.[12] Large tissue samples can be homogenized into a paste using a tissue grinder or food processor.[13,14] With both of these approaches, care should be taken to ensure that the marker residue is not lost due to thermal or enzymatic degradation during the sample processing. As an alternative to techniques that allow the samples to thaw during processing, cryogenic blending provides for grinding the frozen sample. Both liquid nitrogen[15] and dry-ice[16,17] have been used to maintain the samples frozen while they are being processed. Milk samples are generally blended with a mixer or shaken vigorously prior to being sub-divided into a size appropriate for analytical assay.[18] Eggs are generally cracked, and the yolk and white are blended into a uniform mass using a blender. With all of these approaches, care should be taken to ensure that equipment which comes into contact with the sample is cleaned between each sample to avoid cross-contamination. At the time of homogenization, weighing several appropriately sized aliquots of the sample to avoid the need to thaw the sample if further tests are needed may be advantageous.

2.2 Stability

Stability is a critical variable that must be considered as part of method development. When considering stability within the method, reviewing the available stability data for the analyte is very helpful. Information on the stability of the analyte in aqueous

solutions or of metabolic stability is often readily available and can be predictive of potential stability problems in a method. For example, Ali *et al.*[19] showed that the general thermal instability of *N*-methyl carbamate pesticides was also seen as a significant loss of the compound in tissue samples during storage. Several parameters must be considered to ensure that the analyte is appropriately stable at each step of the method. The FDA Guideline 'Bioanalytical Method Validation'[20] lists the parameters long-term tissue stability, short-term tissue stability, freeze and thaw tissue stability, stock solution stability, and post-preparative extract stability as important to consider. Long-term tissue stability is generally evaluated in a range between −20 and −70 °C for several months, if possible. Short-term tissue stability is evaluated at room temperature and is intended to cover the time necessary to process the samples through the procedural steps of the method. Freeze and thaw stability is evaluated based upon the realization that tissue samples often go through several cycles of freeze and thaw before final analysis. The Guideline suggests that three freeze and thaw cycles are generally adequate to evaluate this parameter. Stock solution stability and post-preparative extract stability evaluate the compound in the solutions used in the method.

3 Extraction and sample preparation

Once the sample has been processed in such a way as to maintain residue stability, to prevent cross-contamination, and to ensure homogeneity, strategies to extract the drug from the tissue and to isolate the drug residue from potential interferences must be evaluated. The following sections will review these two concepts separately.

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3.1 Extraction

3.1.1 Solvent extraction

By far the most common approach to removing the drug residue from the tissue sample matrix is to select an appropriate solvent into which the drug residue will selectively partition. For many methods, devices such as high-speed mixers/blenders,[21] sonicators,[22] or a stomacher apparatus[23] can aid with the kinetics of the solvent extraction process. High-speed mixers/blenders use a rapid shearing action to break the tissue into very small pieces that allow exposure to the solvent. Sonication probes or ultrasonic baths use high-frequency ultrasonic waves to disrupt the tissue matrix. Sonication ruptures tissue cell membranes, releasing the cellular contents into the solvent. This aspect of sonication is of greatest utility for highly aqueous extracts because organic solvents will also rupture the tissue cell membranes. A stomacher is a blending device in which the tissue and solvent are placed into a plastic bag and forced compression is used to thoroughly the mix the contents. The stomacher has the advantage that each sample is contained, and the risk of cross-contamination is reduced. The most appropriate device to use for a given method is the one that gives the highest drug recovery while leaving behind interfering components from the tissue matrix. As McCracken *et al.*[23] pointed out, some compounds are easily extracted and

may not require vigorous methods which would only facilitate the removal of a greater amount of extraneous interference.

The most critical decision to be made is the choice of the best solvent to facilitate extraction of the drug residue while minimizing interference. A review of available solubility, $\log P$, and pK_a/pK_b data for the marker residue can become an important first step in the selection of the best extraction solvents to try. A selected list of solvents from the literature methods include individual solvents (n-hexane,[24] dichloromethane,[25] ethyl acetate,[26] acetone,[27] acetonitrile,[28] methanol,[29] and water[22]); mixtures of solvents (dichloromethane–methanol–acetic acid,[30] isooctane–ethyl acetate,[31] methanol–water,[32] and acetonitrile–water [33]), and aqueous buffer solutions (phosphate[34] and sodium sulfate[35]). Hexane is a very nonpolar solvent and could be chosen as an extraction solvent if the analyte is also very nonpolar. For example, Serrano *et al.*[24] used n-hexane to extract the very nonpolar polychlorinated biphenyls (PCBs) from fat, liver, and kidney of whale. One advantage of using n-hexane as an extraction solvent for fat tissue is that the fat itself will be completely dissolved, but this will necessitate an additional cleanup step to remove the substantial fat matrix. The choice of chlorinated hydrocarbons such as methylene chloride, chloroform, and carbon tetrachloride should be avoided owing to safety and environmental concerns with these solvents. Diethyl ether and ethyl acetate are other relatively nonpolar solvents that are appropriate for extraction of nonpolar analytes. Diethyl ether or ethyl acetate may also be combined with hexane (or other hydrocarbon solvent) to create an extraction solvent that has a polarity intermediate between the two solvents. For example, Gerhardt *et al.*[31] used a combination of isooctane and ethyl acetate for the extraction of several ionophores from various animal tissues.

Acetonitrile and methanol are very popular extraction solvents because of their intermediate polarity, especially with animal health drugs that tend also to be of intermediate polarity necessary for them to be orally absorbed. There are differences between acetonitrile and methanol as extraction solvents due to the hydrogen bonding characteristics of methanol. This is particularly true with respect to methanol being able to solvate proteins differently from acetonitrile. Also, acetonitrile[33] and methanol[32] can be combined with water to create a more polar solvent. Water is a highly polar solvent. Because of its polarity and hydrogen-bonding ability, water will tend to extract a great deal of extraneous tissue matrix, including proteins. Also, metabolites are generally more polar than the parent compound and will tend to be more soluble in water. On the other hand, water may be the only alternative for very polar analytes. For example, Cherlet *et al.*[36] used water to extract gentamicin, which is highly polar owing to multiple primary and secondary amino groups. If water is used as the extraction solvent, precipitating proteins with trichloroacetic acid,[14,36] oxalic acid,[37] or zinc[38] may be helpful in reducing the matrix load in the extract.

If the analyte contains either an acidic or a basic functionality, adjusting the pH of the extraction solvent to make the analyte either ionic or nonionic may be advantageous. To make an analyte that contains an acidic or basic functionality nonionic for extraction into a nonpolar solvent, a small amount (5% or less) of an organic acid (such as acetic acid or trifluoroacetic acid) or organic base (triethylamine) along with methanol (about 10%) can be added to diethyl ether or ethyl acetate.[39] Conversely, buffered solutions can be used to control the pH precisely in such a way as to control the charge on an analyte and thus improve its extraction efficiency into polar solvents.

As an example, Bergwerff *et al.*[40] used citrate buffer (pH 4.0) to protonate the amines in spectinomycin and facilitate its extraction into water.

However, when considering the use of acid or base in organic solvents for sample extraction, care must be taken to avoid potential artifacts that may arise from side reactions. For example, methylation of active hydroxyl groups or acidic functions on the analyte may sometimes occur when acidic methanol is used as the extractant. Another example is acetylation of an active alcohol on the analyte following partition of the analyte into ethyl acetate from aqueous solution acidified with glacial acetic acid.

Several extraction techniques have also been described that use enzymatic or chemical reactions to improve extraction efficiency. A technique that has been used to increase the overall recovery of the marker residue is enzymatic hydrolysis to convert specific phase II metabolites (glucuronides or sulfates) back into the parent residue. Cooper *et al.*[10] used a glucuronidase to increase 10-fold the concentration of chloramphenicol residues in incurred tissue. As an example of a chemical reaction, Moghaddam *et al.*[41] used Raney nickel to reduce thioether bonds between benomyl and polar cellular components, and as a result achieved a substantially improved recovery over conventional solvent extraction. In choosing to use either of these approaches, thorough characterization of the metabolism in the tissue sample must be available.

3.1.2 Supercritical fluid extraction

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Supercritical fluid extraction (SFE) is a technique in which a supercritical fluid [formed when the critical temperature (T_c) and critical pressure (P_c) for the fluid are exceeded simultaneously] is used as an extraction solvent instead of an organic solvent. By far the most common choice of a supercritical fluid is carbon dioxide (CO_2) because CO_2 has a low critical temperature ($T_c = 31.1\,^{\circ}C$), is inexpensive, and is safe.[42] SFE has the advantage of lower viscosity and improved diffusion coefficients relative to traditional organic solvents. Also, if supercritical CO_2 is used as the extraction solvent, the solvent (CO_2) can easily be removed by bringing the extract to atmospheric pressure.[43] Supercritical CO_2 itself is a very nonpolar solvent that may not have broad applicability as an extraction solvent. To overcome this problem, modifiers such as methanol can be used to increase the polarity of the SFE extraction solvent. Another problem associated with SFE using CO_2 is the co-extraction of lipids and other nonpolar interferents. To overcome this problem, a combination of SFE with SPE can be used. Stolker *et al.*[44] provided a review of several SFE/SPE methods described in the literature.

3.1.3 Accelerated solvent extraction

Accelerated solvent extraction (ASE) is a technique which attempts to merge the beneficial solvation properties of SFE with traditional organic solvents. Specifically, the sample is placed in an extraction vessel which can withstand high pressures while being maintained at a constant temperature. Extraction is carried out by pumping the extraction solvent through the samples for a limited time. As an example of the use of ASE, Richter and Covino[45] extracted PCBs from a 10-g fish tissue sample with hexane

at 125 °C and 1500 psi. Given the high temperatures achieved with ASE, the analyte stability must be monitored or known to ensure that there is no degradation even though the analyte is only exposed to the high temperatures for a short period of time (minutes). The advantage of ASE is usually a reduction in extraction time and solvent volume compared with similar extraction methods such as Soxhlet extraction.[46] Another technique that has been used on a limited basis but has some similarity to ASE with respect to extraction under high temperature and pressure is microwave-assisted extraction (MAE). Weichbrodt *et al.*[47] showed that similar results were obtained by MAE and ASE for the extraction of organochlorine compounds from fish tissue.

Finally, the benefits of using either radiolabeled analyte or incurred residue as a tool for the evaluation of any of these extraction procedures should be mentioned. Radiolabeled analyte spiked into control tissue can be useful for the evaluation of the recovery of an extraction process by itself without being complicated by the other steps of the method. Incurred radioactive residues can also be very useful for the purpose of evaluating recovery, but a thorough understanding of the amount of marker residue in the incurred sample must be determined prior to this evaluation because the extraction efficiency of total residue would not be expected to be the same as the marker. The use of nonradiolabeled incurred residue can also be used to evaluate recovery differences between incurred and spiked residues. An accepted way to demonstrate the similarity between incurred and spiked residues is to evaluate the results generated through multiple extractions of the same incurred tissue sample matrix and compare the results with those expected from spiked samples. All of these techniques can provide much needed information to maximize the recovery of the marker residue and, as a result, improve the sensitivity and reliability of the method. The goal of extraction is to maximize recovery while minimizing extraneous components that may interfere with subsequent steps. This balance between competing principles may ultimately require a compromise to obtain the best extraction procedure.

3.2 Sample preparation

Sample preparation is a general term that describes steps of the analytical procedure that separate the analyte of interest from other components of the sample extract that could interfere with detection. Considerable effort in determining the most appropriate sample preparation procedure is generally time well spent in terms of both improved method performance and possible time savings associated with the running of routine samples. Stolker[48] made this point very well by indicating that 50–75% of the total analysis time is taken up in sample preparation. Forethought given in designing a rugged, simple sample preparation procedure could then produce considerable savings when multiplied by a large number of sample analyses that may be required in the future. Stolker analyzed the sample preparation procedures outlined in 200 publications. His analysis demonstrated that SPE was the most popular technique and was used in 46% of the methods. Other techniques included liquid–liquid partition (31%), SFE (10%), immunoaffinity extraction (5%), dialysis (5%), and matrix solid-phase dispersion (1%). A summary discussion of these techniques here will provide an outline for the review of sample preparation in the subsequent sections.

3.2.1 Solid-phase extraction

SPE is a sample preparation approach in which the sample extract is passed through a solid sorbent to take advantage of differing partition coefficients of the analyte and the possible matrix interference. There are primarily two purposes for using SPE. First, interfering compounds can be eliminated from the sample extract that could produce complications in the subsequent separation and detection steps of the method. Second, a dilute sample extract can be concentrated to increase sensitivity. Generally, but not exclusively, SPE involves three distinct steps: applying the sample extract to the SPE cartridge in such a way as to retain the analyte, washing the SPE cartridge with solvents that remove interference but still retain the analyte, and eluting the analyte from the column.

The most important step in developing an SPE procedure is the selection of the correct SPE sorbent for the analyte being investigated. A number of SPE sorbents are commercially available and use the following retention mechanisms to separate the marker residue from matrix components: normal-phase, reversed-phase, ion-exchange, and mixed-mode. Normal-phase SPE uses a polar sorbent in combination with nonpolar solvents to facilitate the isolation of the analyte. Typical normal-phase SPE sorbents are silica,[49] alumina,[28] aminopropyl,[50] and cyanopropyl. Conversely, reversed-phase SPE uses a nonpolar sorbent in combination with polar solvents. Reversed-phase sorbents can be derived from two different support types, silica and polymeric resins. Silica supports are highly porous and have a large surface area to improve the loading capacity, but secondary interactions from residual silanols can produce inconsistencies in performance for analytes that contain polar groups such as amines or carboxylic acids. Typical silica-based reversed-phase SPE sorbents include octadecyl (C_{18}), [26,51] octyl (C_8), and phenyl. Resin-based reversed-phase SPE cartridges use a polymer backbone to support the sorbent. Polymeric SPE cartridges are generally more nonpolar than silica-based cartridges because of the polarity difference in the backbones. An example of a polymeric SPE cartridge is poly(styrene–divinylbenzene).[37] Ion-exchange SPE uses interactions between oppositely charged ions as the mechanism of analyte retention. Ion exchange can be cationic with sorbents such as sulfonic acid[21] or carboxylic acid[40] ligands and anionic with sorbents such as alkylamine ligands.[50] More recently, SPE cartridges that combine reversed-phase and ion-exchange mechanisms have been developed to produce improved selectivity. This so-called mixed-mode SPE[52,53] can be selected for anionic or cationic analytes.

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When selecting the most appropriate SPE cartridge to use, several factors may influence the decision. One consideration is the chemical properties of the marker residue such as polarity and pK_a/pK_b. Polar analytes will be retained to a greater extent on a normal-phase cartridge, whereas a reversed-phase cartridge will better retain nonpolar analytes. The pK_a/pK_b of the analyte will dictate if ion-exchange or mixed-mode mechanisms are even possible for the analyte. Another consideration is the polarity of the extraction solvent that was used to remove the drug from the tissue. For example, if the extraction solvent is nonpolar, then the use of a polar sorbent would be the most appropriate; otherwise, the extraction solvent would have to be removed prior to applying the sample to the SPE. Also, the strength of the extraction solvent may not be weak enough to retain the analyte on the SPE sorbent during the application phase, and the addition of a weaker solvent may be necessary. An

example of this is the retention of tilmicosin on a C_{18} SPE sorbent after extraction with methanol.[12] In this case, an additional 75 mL of water was added to the 20 mL of methanol extract to retain tilmicosin on the SPE cartridge. A third consideration is the nature of the dissolved tissue matrix in the sample extract. If the dissolved tissue matrix is either more or less polar than the analyte, then the wash and elution steps may be tailored to separate the analyte from those interfering substances. Also, the tissue matrix may dictate the size of the SPE cartridge to be used. A great deal of dissolved tissue matrix could overload an SPE cartridge of inadequate size. Finally, mixed-mode SPE may offer significant advantages for analytes that contain an ionic group. In this instance, the marker residue can be alternately retained by reversed-phase and ion-exchange mechanisms while the matrix components are washed off the SPE cartridge.

A technique that attempts to combine the extraction and SPE into a single step is matrix solid-phase dispersion (MSPD).[43,54] In this technique, a nonpolar (such as C_{18}) SPE sorbent is blended directly into tissue matrix, the mixture is packaged into an SPE cartridge, and the cartridge is eluted like a typical SPE cartridge. The advantage of MSPD is reduced sample size and increased efficiency due to a reduced number of steps.

Another subset of SPE is immunoaffinity extraction, in which an antibody specific to the analyte is incorporated into the SPE sorbent.[55] This technique is very selective to the analyte and would be very effective in separating the marker residue from tissue-related matrix components. Disadvantages of immunoaffinity extraction are the need to develop a specific antibody-based SPE for each analyte. This approach holds promise for the future as the development of antibody-based methods becomes more commonplace.

3.2.2 Liquid–liquid partition

Liquid–liquid partition (LLP) is a sample preparation technique that takes advantage of the difference in polarity that occurs when analytes containing acidic or basic functional groups transition between an ionic and a neutral form. In a general LLP procedure, the pH of the sample extract is adjusted so that most of the analyte molecules remain in the sample extract while being washed with a solvent that is not miscible with the sample extract. By doing this, extraneous material from the extract solution is removed while leaving the analyte behind. Then, the pH is adjusted so that the analyte would have the opposite charge (neutral or ionic), and the sample extract is again washed with a solvent that is not miscible in the sample extract solution. This time the analyte is partitioned into the wash solution leaving behind additional interfering compounds. In LLP, a significant polarity difference must be maintained between the two solvent phases so that the solvents will not have appreciable solubility in each other. This will maximize the retention of the analyte in the phase of interest and minimize emulsions. To achieve this, the extraction solvent polarity may have to be enhanced by addition of either a polar or a nonpolar solvent. n-Hexane might be added to a nonpolar sample extractant solution to achieve greater hydrophobicity, or a salt (e.g., NaCl) could be added to an aqueous solution to create a solution of greater ionic strength. An example of LLP is in the determination of azamethiphos by Pfenning *et al.*,[26] who used hexane–water LLP to remove fats from the sample extract.

They also used a combination of LLP and SPE, which might be a good strategy if the sample extract has a high load of matrix interferents. Another example of LLP is provided by Furusawa,[56] who used hexane–acetonitrile LLP to remove interfering compounds from the sample extract solution in the determination of spinamycin in chicken eggs and tissue. LLP will likely play a reduced role in future methods owing to the emergence of mixed-mode SPE because both techniques are based upon similar principles, but mixed-mode SPE offers the advantage of greater selectivity (theoretical plates), less labor intensity, and greater sample throughput. LLP will probably not be completely replaced, however, because of binding or stability problems associated with silica supports for some compounds.

3.2.3 Ultrafiltration and on-line dialysis

Until this point, the sample preparation techniques under discussion have relied upon differences in polarity to separate the analyte and the sample matrix; in contrast, ultrafiltration and on-line dialysis rely upon differences in molecular size between the analyte and matrix components to effect a separation. In ultrafiltration, a centrifugal force is applied across a membrane filter which has a molecular weight cut-off intended to isolate the analyte from larger matrix components. Furusawa[57] incorporated an ultrafiltration step into his separation of sulfadimethoxine from chicken tissue extracts. Some cleanup of the sample extract may be necessary prior to ultrafiltration, or the ultrafiltration membranes can become clogged and ineffective. Also, one must ensure that the choice of membrane filter for ultrafiltration is appropriate in terms of both the molecular weight cut-off and compatibility with the extraction solvent used.

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On-line dialysis also separates the analyte from tissue matrix based upon molecular size, but in this case, the sample extract is passed over a membrane filter through which the analyte (and other low molecular weight compounds) is diffused into a second solvent on the other side of the membrane filter. Usually, the second solvent is then concentrated on to an SPE column to minimize the dilution effect that is caused by the dialysis process. Agasoester[58] used on-line dialysis to separate oxytetracycline from muscle, liver, milk, and egg tissue matrix components. A problem encountered with on-line dialysis is the inability of analyte molecules that are bound to proteins in the sample extract to pass through the membrane filter.[59] Problems with membrane clogging are reduced with on-line dialysis compared with ultrafiltration because no external force is being applied to bring the analyte across the membrane filter.

4 Separation and detection

A survey of the literature with a key phrase ‘tissue residue analysis’ yielded a distribution of separation and detection techniques as outlined in Table 2. LC with either UV or fluorescence detection was the most common separation and detection technique, representing 61% of the citations. The results are an indication of the maturity of LC as a common, well-understood technique. The second most commonly used technique cited in the literature (13%) was GC with either a mass-selective or electron capture detector. GC is also a mature technology and a good choice owing to the

Table 2 Survey results of separation and detection methods used in tissue residue analysis

Detection technique	Proportion of citations (%)
LC	61
GC	13
Immunoassay	7
LC/MS	13
TLC[a]	5

[a] TLC = thin-layer chromatography.

greater selectivity that occurs with capillary GC methods. GC is not as common a technique as LC because the trend in drug and pesticide molecules has been toward higher molecular weight, nonvolatile molecules. Immunoassay methods represented 7% of the citations and are an area that would be expected to grow in the future. Immunoassay is particularly applicable as a screening method owing to its ability to be automated. Finally, LC in combination with mass spectrometry (MS) is a technique that represents 13% of the citations in this survey. Although less common than LC and equivalent to GC in this survey of the literature, liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) are likely to experience future growth as the availability of the instruments becomes more commonplace. The reason for this trend is the improvement in selectivity that LC/MS and LC/MS/MS bring with respect to sample matrix components. The following sections will look into each of these techniques (LC, GC, immunoassay, and LC/MS) individually in more detail as applied to tissue residue analysis.

4.1 Liquid chromatography

4.1.1 Separation in liquid chromatography

LC is a technique in which the mobile phase (solvent) is passed over a stationary phase (usually a silica-based support) at high pressure. The separation of analyte molecules from the matrix components is determined by the difference in partition between the mobile and stationary phases. Reversed-phase LC is the most common separation technique among tissue residue methods.[27,34,60] Reversed-phase LC is a mature technology with a wealth of literature and books that describe the technique in detail. Books such as 'Practical HPLC Method Development' by Snyder *et al.*[61] give a very detailed treatment of LC theory and practice that will not be repeated here. There are, however, a few trends in reversed-phase separations that are worth mentioning because of some potential advantages for tissue residue methods. In general, trends in reversed-phase separations are based upon two inherit limitations[62] associated with using silica as a column support. The first limitation is the presence of residual silanol groups on bonded phases that can create secondary interactions in addition to the primary reversed-phase separation. Since these secondary interactions have slower kinetics than the primary reversed-phase interaction, the presence of residual silanols can produce significant peak broadening. Residual silanols work by an ion-exchange

mechanism, and so drugs that contain carboxylic acids or amines are susceptible to this type of peak broadening. This is particularly important, as a significant number of drugs have either a carboxylic acid, an amine, or both functional groups. The second limitation to silica-based columns is the available pH range. In silica-based LC columns, pH values of <2 or >7 are unacceptable owing to instability of the support at these pHs. For amines and other basic functional groups, this limited pH range translates into the compound always being ionic in the mobile phase and not as easily partitioned into the stationary phase.

An approach to addressing the limitations of silica-based reversed-phase columns is to introduce additives to the mobile phase that compensate for the problems. Vervoort *et al.*[62] described three specific mobile phase approaches that can greatly improve separation efficiencies for amine- or carboxylic acid-containing compounds: (1) adjusting the pH to the range 2–3, (2) adding blocking agents (tertiary amines or quaternary ammonium salts), and (3) adding ion-pairing reagents. A second approach for addressing the limitations of silica-based columns is to dispense with the use of silica as a support altogether and instead use a polymeric-based reversed-phase column. Posyniak *et al.*[37] used a polymeric reversed-phase separation for tetracyclines, which contain a tertiary amine. Limitations to using polymeric reversed-phase columns are a reduced column lifetime and lower efficiency compared with silica-based supports. A third approach to the limitations of silica reversed-phase columns is to use columns that have a polar group incorporated into the stationary phase.[63] The benefit of this approach is the improved solvation of the stationary phase when highly aqueous mobile phases are used. For example, amino groups are generally protonated at the pHs used in reversed-phase separations and as such may require a high percentage of water in the mobile phase to retain the compound effectively on the column. When a high percentage of water is used in the mobile phase, there is a tendency for the stationary phase to collapse and be less effective at partitioning. Incorporating polar groups into the stationary phase will prevent this from happening. The final approach to solving the limitation of silica reversed-phase columns is to use silica phases that are specifically designed to allow for pH values >7.[64,65] For amines, these columns have the advantage of operating in a pH range that will allow the amine functional group to be nonionic and partition more effectively into the stationary phase. These approaches, potentially used in concert, can provide the opportunity to effect the best separation of amines or carboxylic acid-containing compounds.

Descriptions of separations on supports other than the reversed-phase type are very limited in the literature, but a few examples are presented here for completeness. Furusawa[56] used a normal-phase separation with an aminopropyl column to determine spiramycin in eggs and chicken tissue. Hornish *et al.*[66] separated spectinomycin residues from bovine tissue matrix components using ion-exchange LC separation. Bidlingmeyer and Ekmanis[67] demonstrated the use of small molecule gel permeation chromatography to separate herbicide and pesticide residues from tissue matrix components. Finally, the benefits in sensitivity that can be achieved by any of these separation techniques through the use of narrow-bore columns should be mentioned. For example, Porter and Johnston[68] achieved an improvement in sensitivity of up to fivefold for the determination of tetracyclines by decreasing the column diameter from 4.6 to 2.1 mm.

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4.1.2 Detection in liquid chromatography

Once the analyte has been separated from the matrix in LC, the best approach to the detection of the molecule must be determined. This section will discuss the detection techniques of ultraviolet/visible (UV/VIS), fluorescence (FL), and electrochemical (EC) detection, with MS being addressed separately in Section 4.2. When deciding on the most appropriate detector for an LC separation, the appropriate chemical data on the analyte should be collected by using a spectrophotometer, fluorimeter, and potentiometer.

Using a spectrophotometer, the wavelength of maximum absorbance should be determined for the analyte and should serve as the basis for the choice of wavelength in the separation. For example, Liguoro *et al.*[32] used tylosin's wavelength of maximum absorbance of 280 nm for their UV detection method. With UV detection, the choice of the best wavelength may also be dependent upon the UV absorbance of the extract matrix components. Usually a longer wavelength will result in a smaller interference, particularly for wavelengths between 200 and 300 nm. Hence choosing a wavelength that is larger than the maximum wavelength may help to reduce potential interference. As an example of this, the detection of diacerhien[69] in plasma is improved by choosing a detection wavelength of 432 nm rather than the maximum wavelength of 258 nm because of greater interference at 258 nm.

For FL detection, maximum emission and excitation wavelengths are determined using a fluorimeter. Stoev[70] used fluorescence detection to analyze for closantel (excitation at 335 nm, emission at 510 nm) residues in animal tissue.

Electrochemically active compounds can be evaluated using a potentiometer to generate a cyclic voltammogram for the analyte. Cyclic voltammetry will allow the analyst to determine whether the compound can be oxidized or reduced, to choose the appropriate potential to use in the electrochemical detector, and to establish whether oxidation or reduction is irreversible. Irreversible oxidation or reduction of the analyte could be predictive of problems with electrode poisoning and reduced sensitivity of the electrochemical detector over time. Turberg *et al.*[71] used EC detection at an applied potential of +600 mV to analyze for ractopamine.

When deciding among the three techniques, the first consideration is the sensitivity as determined by the mass of the analyte that can be detected from standard solutions. The sensitivity is specific to the structure of the compound and in some cases may not be obvious. Therefore, serial dilutions of standards should be made to test the sensitivity, to be assured of choosing the most sensitive detector. The second consideration is matrix interference from the sample extract that co-elutes in the retention window of the analyte. Therefore, testing a blank control sample extract is necessary to assess background interference of the matrix. FL detection may offer an advantage here, because a high percentage of tissue sample matrix components do absorb UV radiation but do not fluoresce. A final consideration is the ruggedness of the analytical techniques. In general terms, UV and FL detection offer a considerable advantage over EC detection with respect to ruggedness. In the experience of the authors, electrochemical methods can be very sensitive for certain compounds but are generally less reproducible with respect to detector response over time when compared with UV or FL. Also, a greater level of experience is needed with EC than with UV or FL detection to troubleshoot problems.

At this point, the analyte may not be amenable to UV, FL, or EC detection. In this case, the best course of action may be to choose LC/MS (see Section 4.2). However, one other option is to use a pre-[14] or post-column[49] derivatization step to increase the detectability of the analyte with respect to FL or UV. Fluorescent or UV labels are available for carboxylic acids,[49,72] amines,[66,73] phenols, and thiols. The decision to use pre- or post-column derivatization is predicated upon the functionality of the analyte available for derivatization and the rate and extent of the reaction between each derivatizing agent and the analyte.

4.2 Liquid chromatography/mass spectrometry

LC/MS is a separation and detection technique that has grown dramatically in popularity in recent years owing to advantages in selectivity and sensitivity over conventional methods. The use of LC/MS methods for monitoring of violative residues includes determinative methods for quantitation and confirmatory methods for structural verification. LC/MS theory is outside the scope of this article, but 'A Global View of LC/MS' by Willoughby *et al.*[74] is a recommended reference for general and background information on LC/MS techniques. The advancement of LC/MS as a widely used technique can be attributed to the development of interfaces that allow for atmospheric pressure ionization,[75] specifically electrospray and atmospheric pressure chemical ionization interfaces. A number of review articles[76,77] have been published on the application of LC/MS for analysis of tissue residue samples. Popular commercial instrumentation for LC/MS includes single and triple tandem quadrupole, quadrupolar ion trap, and time-of-flight based designs. Triple tandem quadrupole instruments (LC/MS/MS) in particular have emerged as somewhat of a convention in the characterization and quantitation of residues in food animal tissues because they offer significant advantages in terms of specificity and sensitivity. For example, Furst[77] vastly improved sensitivity and selectivity in the detection of metonidazole in turkey tissue by LC/MS/MS when compared with LC/MS alone. As a result, recent literature[66,78,79] published on tissue residue methods has trended towards the use of LC/MS/MS as a preferred technique for development of regulatory determinative and confirmatory MS methods. Another advantage associated with the use of MS methods (both LC and GC) is the opportunity to use isotopic (^{2}H or ^{13}C) internal standards in quantitation. Isotopic internal standards behave like the marker residue with respect to chemical properties but are separated in the mass domain. For example, Schloesser *et al.*[80] used a ^{13}C-labeled benzylpenicillin as an internal standard for the determination of benzylpenicillin in tissue analysis.

Mass spectrometry [either gas chromatography/mass spectrometry (GC/MS) or LC/MS] is the method of choice for confirmatory analytical methods. Confirmatory methods can provide structural verification by using either full-scan spectra or the analysis of several structurally specific ions. Full-scan spectra are used in cases of sufficiently high signal-to-noise ratio, allowing structural verification through a unique 'fingerprint' mass spectrum. The second method for confirmation of the presence of a target analyte involves the monitoring of several structurally specific fragment ions that are generated reproducibly and are consistent with an authentic standard in terms of observed mass-to-charge ratio (m/z) and ratios of respective ion intensities.

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This selected ion monitoring (SIM) approach typically has greater applicability in cases where sensitivity is more of a concern. Kiehl and Kennington[81] developed a swine liver confirmatory method for tilmicosin that confirmed structure based upon monitoring a parent ion and two additional structural fragment ions. A discussion of the validation requirements for confirmatory methods is provided in Section 6.

When using LC/MS for either determinative or confirmatory methods, several practical items must be considered. First, the mobile phase and buffers must be volatile and compatible with LC/MS interfaces. This is particularly important if existing methodology is being converted to the LC/MS platform. Second, the characteristics of the molecule such as polarity and functional groups must be taken into consideration with the choice of mobile phase, ionization, and analyzer with MS techniques. Third, the analytical separation must be optimized to ensure that the matrix and marker analyte are adequately separated. A common misconception with LC/MS techniques is that the separation is unimportant because of the improved selectivity with LC/MS methods. The presence of co-eluting matrix components can result in suppressed ionization and reduced sensitivity. For example, Matuszewski *et al.*[82] observed significant loss of sensitivity from samples analyzed for finasteride owing to ion suppression from endogenous compounds. When a method cleanup and better chromatographic separation were employed, the sensitivity of finasteride detection in the sample extracts was restored. Further, structurally similar metabolites in tissues may also elute close to the target analyte and generate fragment ions of similar m/z that can interfere with SIM analysis. A fourth misconception about LC/MS is that its use will automatically result in an improvement in sensitivity. Since the sensitivity of LC/MS methods is largely dependent on the ionizability of the analyte, the sensitivity of LC/MS can vary greatly between analytes. Other detection techniques such as UV or FL may have greater sensitivity than LC/MS, particularly if the analyte is highly aromatic with few ionizable functional groups. In the final analysis, the disadvantages of LC/MS, such as increased cost of instrumentation and more specialized expertise required of the analyst, are in many cases outweighed by advantages in selectivity and sensitivity that LC/MS techniques provide.

4.3 Gas chromatography

4.3.1 Separation in gas chromatography

GC is a mature technology that has been applied to the determination of pesticides and drugs[83] in foodstuffs for many years. A thorough review of GC theory and practice is provided in 'Chromatographic Methods' by Braithwaite and Smith.[84] The trend in recent years away from GC has little to do with the performance of GC methods but rather with a shift in pesticide and drug residues to higher molecular weight, nonvolatile compounds. For compounds that are volatile, GC offers an advantage over LC of improved specificity and, in some cases, greater sensitivity. Currently, almost all GC methods are based on wall-coated open-tubular capillary columns because they offer high efficiencies (up to 10 times higher than LC). This efficiency advantage makes GC ideally suited for methods that monitor multiple compound residues. For example, Pfenning *et al.*[39] used a GC method to analyze for chloramphenicol,

florfenicol, florfenicolamine, and thiamphenicol in shrimp tissue in a single method. Factors influencing the choice of the best GC capillary column stationary phase are polarity, boiling point, and thermal stability of the analyte. GC columns are generally subdivided based upon the polarity of the analyte into nonpolar, midpolar, and polar classifications. The boiling point range is a second and equally important factor influencing GC column selection because GC stationary phases are stable only within a certain temperature range. Finally, the film thickness of the stationary phase must be considered to ensure that the column has an appropriate loading to separate the analyte adequately from matrix components. Once the best GC column has been chosen, the method must then be optimized with respect to carrier gas velocity and temperature gradient program. The goal of this optimization is to choose the combination of parameters that provides a rugged, baseline separation of the analyte or analytes in the minimum time. Another point to consider is the possibility of derivatization of the analyte to increase the vapor pressure or improve the thermal stability of the compound. The most common derivatization approaches[84] for GC include silylation,[85] acylation, and alkylation.[86] Derivatization may also be used to introduce functional groups that improve the sensitivity of detection.

4.3.2 Detection in gas chromatography

Once the GC separation has been achieved with the appropriate column, temperature program, and carrier gas velocity, the best GC detection method must be chosen from among flame ionization detection (FID), electron capture detection (ECD), nitrogen–phosphorus detection (NPD), and MS. FID is the most common GC detection method for general use owing to its nearly universal detection capability, but FID is not frequently used for tissue residue analysis because this detection lacks specificity and sensitivity. ECD offers significant sensitivity advantages for compounds containing electronegative elements such as chlorine and fluorine. Since a number of pesticides and herbicides contain halogen atoms, ECD is particularly useful for tissue residue analysis of these halogenated analytes. For example, Pfenning *et al.*[39] used ECD for the detection of chloroamphenicol, florfenicol, florfenicolamine, and thiamphenicol, taking advantage of the halogen atoms in these compounds. NPD is optimized [84] to be sensitive for compounds containing nitrogen and phosphorus atoms with approximately 50 times greater sensitivity for nitrogen and 500 times greater sensitivity for phosphorus than FID. Luo *et al.*[85] used an NPD to analyze for lincomycin in salmon tissues.

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GC/MS can be used to develop either determinative or confirmatory methods. The typical ion source for GC/MS is electron ionization, and the mass analyzers are the same as those used in LC/MS. A common GC/MS approach to determinative methods involves SIM to monitor the selected mass-to-charge ratio of the parent and/or fragment ions. SIM gives a greater signal-to-noise ratio, resulting in greater sensitivity and specificity based upon monitoring a narrow m/z window. Serrano *et al.*[24] used a SIM GC/MS method for the determination of organochlorine and organophosphorus compounds in whale tissue. As with LC/MS, GC/MS confirmatory methods may consist of acquiring and inspecting full-scan spectra or monitoring selected structurally diagnostic ions. Confirmation of sulfathiazole, sulfamethazine, sulfachloropyridazine, and sulfadimethoxine residues was achieved by Matusik *et al.*[86] using a gas

chromatography/tandem mass spectrometry (GC/MS/MS) method for swine and cattle liver analyses. Selection of the best GC detector along with an optimized separation can create a very sensitive and selective method for those analytes which are volatile and thermally stable.

4.4 Immunoassay

Immunoassay-based methods, such as radioimmunoassay or enzyme-linked immunosorbent assay (ELISA), have grown in popularity as tissue residue methods[87] because of increased productivity, improved sensitivity, and improved selectivity with respect to sample matrix components. Extensive discussions of the production of antibodies or of the development of immunoassay methods are outside the scope of this article. Rowe *et al.*[88] described the production of antibodies and the development of an ELISA for halofuginone. The steps that they took to prepare antibodies and develop an immunoassay included (1) preparation of immunogen for halofuginone, (2) preparation and purification of monoclonal antibodies, (3) characterization of the antibodies with respect to affinity and specificity, and (4) development of a competitive enzyme-linked immunosorbent assay (cELISA). The earlier immunoassay methods for tissue residue were developed with polyclonal antibodies which were isolated directly from the animal, but more recent methods use monoclonal antibodies which are derived from cells that produce the desired antibody. Monoclonal antibodies[89] are almost a necessity for the development of regulatory methods because a readily available immunoreagent is needed. Immunoassay methods may be adapted to a 96-well format to increase sample throughput and, hence, are ideally suited for use as screening methods to check for potentially violative residues prior to determinative and confirmatory testing. For example, Mitchell *et al.*[90] describes the use of a cELISA to screen for β-lactam residues in bovine tissues. Screening methods may also be adapted to plasma[88] or urine to allow for screening of drug residues prior to animal slaughter.[91] Immunoassay methods are usually very sensitive because the detection is not based directly on a chemical property of the analyte but rather on a detection property of a tag or label molecule. Specificity with respect to tissue matrix is generally improved because of the inherent specificity of antibody binding. This may allow the sample extract to be purified to a lesser extent than with chemical methods, but the sample extract must be in an appropriate solvent (usually an aqueous buffer) to ensure appropriate binding. Selectivity with respect to structurally similar compounds such as metabolites or analogs can be of concern with immunoassay methods as the binding may not be specific with respect to structurally similar compounds.[92] A check for cross-reactivity to structurally similar compounds is mandatory when validating an immunoassay method. A similar issue with immunoassay methods is the correlation between immunoassay methods and existing chemical methods. Since the immunoassay methods are dependent upon the availability of antibodies, these methods are not generally developed until later in the registration lifecycle. In most cases, a chemical method may already exist and may have been used in establishing a tolerance. If a new immunoassay method cannot be correlated to an existing chemical method, then the immunoassay will not be accepted as a regulatory method because no link to the tolerance can be established. In some cases, the correlation between immunoassay and chemical methods may not be straightforward since the presence of

metabolites at longer withdrawal times may be detected in the immunoassay method but not the chemical method. Beier *et al.*[93] demonstrated the correlation between their cELISA and existing LC assays to demonstrate the validity of the new assay. As was mentioned earlier, most immunoassay methods in the recent literature address immunoassay as a screening method, but the use of immunoassay for the determinative method is becoming more accepted. For example, Young *et al.*[94] used an ELISA method as the determinative method for the quantitation of spinosad in animal tissue.

5 Data handling and presentation

Data systems for the collection and processing of chromatographic data are commercially available and advanced in their function. Current software can detect and integrate the peak or peaks of interest automatically. If the computer system is being used for peak identification and integration, the peaks of interest must be adequately resolved from interfering peaks to ensure that the peak integration is consistent between samples and standards. An additional feature of chromatographic systems that must be considered is the capability of the computerized laboratory information management system (LIMS) to store, archive, and retrieve data in a manner that ensures the integrity and security of that information. Computer system validation is important in ensuring that the capability of the software is adequate to provide security against loss or alteration of data. An additional consideration with chromatographic data systems is system suitability parameters. System suitability criteria are chromatographic parameters unique to each method that ensure that the method is functioning in an acceptable manner. Typical system suitability parameters could include peak resolution, peak tailing or asymmetry, and precision. Computer systems that can process the system suitability parameters for the method could provide savings in time and improvements in consistency.

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A fundamental deficiency faced with commercially available MS instrumentation is that hardware development is advancing faster than the capability to acquire and process data, particularly in a high-throughput environment. As a result, consideration must be given to whether or not the software is appropriate to meet the requirements of the method. A high level of multi-tasking may be necessary to acquire, process, and store data simultaneously. This is particularly true as instrumentation tends toward high data acquisition rates (megahertz), high resolution capability, and large data file storage. Computer system validation is critical to ensuring that the computer system is sufficient to address the method requirements.

Immunoassay data systems are influenced by the need for high-throughput sample analysis and the nature of ELISA response curves. Since ELISA methods are applicable to running samples in a high-throughput (96-well plates) format, this format offers considerable advantages over detection methods that run sequentially. However, the value of these time savings can only be realized if one has the ability to acquire, store, and interpret these data in an efficient way. ELISA data systems should be organized around a high-throughput format so that the efficiencies can be fully realized. Second, the method should be designed around the nonlinearity of the ELISA response curve. An adequate number of standards should be included in the response curve to characterize the response fully. Also, the method response curve should be designed

around the tolerance as much as possible to ensure that the maximum response difference is achieved in the vicinity of the tolerance.

6 Method validation

6.1 Regulatory guidelines

Once the determinative or confirmatory method has been developed to take full advantage of the chemical properties of the analyte molecule, a study is necessary to prove that the method is valid. Criteria for method validation are outlined in guidelines from the US FDA,[95] US EPA,[96] and EU.[97] A summary of the differences in regulatory requirements for method validation is provided in Table 3. The parameters addressed by all of the regulatory guidelines include accuracy, precision, sensitivity, specificity, and practicability.

Method accuracy is defined as the agreement between the measured value and the true value and is usually determined by measuring the percentage recovery of spiked samples. Recovery values of 70–110% are usually desired, although the FDA and the EU allow for wider ranges for analyses at low concentration levels.

Precision is a measure of the agreement between replicate assays and is usually expressed as the coefficient of variation (CV). A CV of 15% or less is desired although, like accuracy, some leniency in this criterion is made for samples at very low concentrations. Also, the regulatory agencies give some consideration to the combined impact of accuracy and precision. For example, a method that has a recovery of less than 70% but a CV of less than 10% might be viewed more favorably than a method with a 90% recovery and a CV of 20%.

Sensitivity is a measure of the smallest concentration that can be either measured [limit of detection (LOD)] or accurately quantitated [limit of quantitation (LOQ)]. In the USA, the method for measuring LOD or LOQ is left up to the method developer. European requirements for determining LOD and LOQ are very specific: the LOD is based on the mean plus three standard deviations for 20 control blank samples, and the LOQ is defined as the lowest concentration giving an acceptable CV.

Specificity is a measure of how selectively the analytical method measures the marker compound in the presence of other compounds. The descriptors used to establish specificity differ depending upon the guideline (see Table 3), but the purpose behind them is the same. In all cases, the method must be demonstrated to have no interference from several (at least five) control animals that represent variation in sex, age, and breed. Further, incurred residue samples or authentic metabolite standards must demonstrate no interference with the marker residue detection. The method must be tested with other approved drugs for the target species to show that no interference exists if these compounds are also present.

Finally, the method must be shown to be practical for use as a routine monitoring method. The method must use commercially available reagents, standards, and equipment. The method must not be too complex or poorly described such that an experienced analytical chemist could not understand or perform the method. Steps of the procedure that are critical should be highlighted in the method so that they can be appropriately controlled. The method must be short enough so that it can be used

Table 3 Comparison of US FDA, US EPA, and EU Committee for Veterinary Medicinal Products (CVMP) method validation requirements

	US FDA[95]	US EPA[96]	CVMP[97]
Accuracy	60–110% ($<100\ \mu g\ kg^{-1}$) 80–110% ($\geq 100\ \mu g\ kg^{-1}$)	70–120%	50–120% ($<1\ \mu g\ kg^{-1}$) 70–110% ($\geq 1\ \mu g\ kg^{-1}$)
Precision	20% ($<100\ \mu g\ kg^{-1}$) 10% ($\geq 100\ \mu g\ kg^{-1}$)	20%	35% ($\leq 1\ \mu g\ kg^{-1}$) 30% (>1 and $\leq 10\ \mu g\ kg^{-1}$) 20%(>10 and $\leq 100\ \mu g\ kg^{-1}$) 15% ($\leq 100\ \mu g\ kg^{-1}$)
Sensitivity (LOD and LOQ)	Based on interferences <10% of tolerance	Defined by sponsor based on variation of control blanks	LOD: defined by mean ±3 standard deviations of at least 20 control blanks LOQ: lowest level giving acceptable precision
Specificity	Method tested with respect to other compounds approved in the target species, co-extracted matrix, and metabolites	Measure and identify the residue in the presence of residues of other pesticides which could reasonably be expected to be present on the commodity	Specificity related to at least any substances which are likely to be present and give a signal, e.g. homologs, analogs, and metabolic products
Practicability	Commercially available reagents, etc. Reasonably experienced analyst Reasonable time No need for unique instrumentation Capable of being performed safely	Does not use exotic equipment Reasonably rapid in execution Practicable without use of extremely hazardous toxic reagents	Commercially available standards, reagents, and equipment Performed safely by trained analyst Complete the analysis of a sufficiently large number of samples in a reasonable time Susceptibility to interference: conditions subject to fluctuation
Confirmatory methods	Minimum of 3 ions Relative abundance 10% external standard (or full spectra) Ions characteristic of molecule	None	Points system based on technique used
Inter-laboratory study	Three laboratories required for approval	One independent laboratory confirmation required	Recommended but not required for approval

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to process a reasonable number of samples within one day. Also, the method should not call for the use of reagents that expose the analyst to undue safety risk.

Confirmatory methods must be sufficiently robust to accurately verify the structure of the analyte. For the US FDA, the validation procedure for confirmatory methods is currently defined by Sphon.[98] Validation criteria include reproducible chromatographic separation, ions chosen to be characteristic of the molecule,

a minimum of three ions, and relative abundances of ions in agreement with an external standard ±10%. As an alternative, if the sensitivity of the method is adequate, a full-scan mass spectrum can be used instead of three confirmatory ions. In Europe, a system is being developed that takes advantage of the improved selectivity that results from LC/MS/MS methodology.

6.2 Inter-laboratory/collaborative studies

The ultimate test of whether a method is acceptable for use in regulatory compliance is whether the method is sufficiently rugged to provide reproducible results in several laboratories during an inter-laboratory or collaborative study. For both US FDA and EPA methods, inter-laboratory ruggedness is required prior to product approval. US EPA methods must undergo a confirmatory trial in one independent laboratory. The independent laboratory must be unfamiliar with the method in terms of both development and use. Successful completion of this inter-laboratory confirmatory trial is reproduction of the original method validation study. US FDA methods must undergo a collaborative study in at least three laboratories prior to approval. Specific guidance with regard to the number and kinds of samples to be tested is provided and includes replicate analysis of both fortified and incurred residues in tissue samples. Finally, mention should be made of AOAC collaborative studies that typically test the method in as many as 8–10 laboratories.[99] These internationally accepted collaborative studies may serve as the ultimate test of the method's performance under a wide variety of inter-laboratory conditions.

7 Conclusion

The development, validation, and use of methods for the determination of drug and pesticide residues is a critical step in the monitoring of appropriate product use. The development of successful determinative and confirmatory methods takes advantage of the unique chemical properties of the molecule to give the best separation and detection. The steps of the procedure must be optimized to ensure the most rugged, sensitive, specific, accurate, and precise method. Further, the method must be thoroughly validated to prove its acceptability. In the end, successful methods gain acceptance in multiple laboratories over long periods of time throughout the world.

References

1. US Food Drug and Cosmetics Act, Sections 409, 512, and 706 (1996).
2. Code of Federal Regulations, Title 21, Part 70, 21 CFR 514.1 (2002).
3. J. Paige, M. Chaudry, and F. Pell, *Chem. Food Borne Hazards Control,* **15**, 45 (1999).
4. Commission Decision 93/257/EEC, *Off. J. Eur. Commun.,* No. L118, 75 (1993).
5. US FDA, General Principles for Evaluating the Safety of Compounds Used in Food-producing Animals, US Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Veterinary Medicine, Washington, DC (1994). Also available on the World Wide Web: **http://www.fda.gov/cvm/guidance/guideline4.html**.

6. L. Friedlander, S. Brynes, and A. Fernandes, *Chem. Food Borne Hazards Control,* **15**, 1 (1999).
7. H. van de Waterbeemd, D.A. Smith, K. Beaumont, and D.K. Walker, *J. Med. Chem.,* **44**, 1313 (2001).
8. E.H. Jansen, R.H. Van Der Berg, R. Both-Miedema, H. Van Blitterswijk, and R.W. Stephany, *Vet. Q.,* **11**, 87 (1989).
9. M.D. Rose, L.C. Argent, G. Shearer, and W.H.H. Farrington, *Food Addit. Contam.,* **12**, 185 (1995).
10. A.D. Cooper, J.A. Tarbin, W.H. Farrington, and G. Shearer, *Food Addit. Contam.,* **15**, 637 (1998).
11. J.G. Beechior and F.J. Bloomfield, *Vet. Rec.,* **149**, 182 (2001).
12. C.M. Stobba-Wiley, J.P. Chang, D.T. Elsbury, J.W. Moran, and R.S. Readnour, *J. AOAC Int.,* **83**, 837 (2000).
13. R.C. Emery, R.V. Gwilliam, K.D. Wilford, and C.J. Threlfall, *Anal. Biochem.,* **91**, 340 (1978).
14. W. Luo, C.Y. Ang, and H.C. Thompson, *J. Chromatogr. B,* **694**, 401 (1997).
15. J.A. Nichols and L.R. Hageman, *Anal. Chem.,* **51**, 1591 (1979).
16. R. Zeisler, J.K. Langland, and S.H. Harrison, *Anal. Chem.,* **55**, 2431 (1983).
17. E.E. Edwin, A.T. Diplock, J. Bunyan, and J. Green, *Biochem. J.,* **75**, 450 (1960).
18. C.M. Stobba-Wiley and R.S. Readnour, *J. AOAC Int.,* **83**, 555 (2000).
19. M.S. Ali, J.D. White, R.S. Bakowski, and E.T. Phillipo, *J. Assoc. Off. Anal. Chem.,* **76**, 1309 (1993).
20. US FDA, Bioanalytical Method Validation, US Department of Health and Human Services, Public Health Service, Food and Drug Administration, Washington, DC (2001). Also available on the World Wide Web: **http://www.fda.gov/cder/guidance/index.html**.
21. L.G. Rush and E.B. Hansen, *J. Chromatogr. B,* **700**, 223 (1997).
22. A.D. Cooper, J.A. Tarbin, W.H. Farrington, and G. Shearer, *Food. Addit. Contam.,* **15**, 645 (1998).
23. R.J. McCraken, D.E. Spence, and D.G. Kennedy, *Food. Addit. Contam.,* **17**, 907 (2000).
24. R. Serrano, F.J. Lopez, and F. Hernandez, *J. Chromatogr. A,* **855**, 633 (1999).
25. A.R. Shoaf and W.C. Carlson, *Weed Res.,* **34**, 745 (1986).
26. A.P. Pfenning, J.E. Roybal, S.B. Turnipseed, S.A. Gonzalas, and J.A. Hurlbut, *J. AOAC Int.,* **82**, 1224 (1999).
27. T.M. Schreier, V.K. Dawson, Y. Choi, N.J. Spanjers, and M.A. Boogaard, *J. Agric. Food Chem.,* **48**, 2212 (2000).
28. G.F. Pang, Y.Z. Cao, C.L. Fan, J.J. Zhang, X.M. Li, and C. Wang, *J. Chromatogr. A,* **882**, 85 (2000).
29. A. Lagana and A. Marino, *J. Chromatogr.,* **588**, 89 (1991).
30. L.J. Kostadinovic, S. Pavkov, and F. Gaal, *Acta Aliment.,* **28**, 311 (1999).
31. G.C. Gerhardt, C.D. Salisbury, and H.C. Campbell, *Food Addit. Contam.,* **12**, 731 (1995).
32. M.D. Liguoro, C. Montesissa, P. Anfossi, and R. Angeletti, *Analyst,* **123**, 1279 (1998).
33. L.G. Rushing and E.B. Hansen, *J. Chromatogr. B,* **700**, 223 (1997).
34. S. Croubels, H. Hermeersch, P. De Backer, M.D. Santos, J.P. Remon, and C. Van Peteghem, *J. Chromatogr. B,* **708**, 145 (1998).
35. L.D. Payne, V.R. Mayo, L.A. Morneweck, M.B. Hicks, and T.A. Wehner. *J. Agric. Food Chem.,* **45**, 350 (1997).
36. M. Cherlet, S. De Baere, and P. De Backer, *J. Mass Spectrom.,* **35**, 1342 (2000).
37. A. Posynick, J. Zmudzki, R.L. Ellis, S. Semeniuk, J. Niedzielska, R. Andrzejak, J. Carbon, E. Jaramin, A. Milian, L. Rodziewics, and G. Zuczek, *J. AOAC Int.,* **82**, 862 (1999).
38. R.P. Maickel, *Crit. Rev. Clin. Lab. Sci.,* **4**, 383 (1973).
39. A.P. Pfenning, J.E. Roybal, H.S. Rupp, S.B. Turnipseed, S.A. Gonzales, and J.A. Hurlbut, *J. AOAC Int.,* **83**, 26 (2000).
40. A.A. Bergwerff, P. Scherpenisse, and N. Haagsma, *Analyst,* **123**, 2139 (1998).
41. M.F. Moghaddam, R.K. Trubey, and J.J. Anderson, *J. Agric. Food Chem.,* **48**, 5195 (2000).
42. E.D. Ramsey, J.R. Perkins, D.E. Games, and J.R. Startin, *J. Chromatogr.,* **464**, 353 (1989).
43. C.C. Walker, H.M. Lott, and S.A. Barker, *J. Chromatogr.,* **642**, 225 (1993).
44. A.A.M. Stolker, M. Sipoli, P.W. Zoontjes, L.A. Van Ginkel, and R.J. Maxwell, *Semin. Food Anal.,* **1**, 117 (1996).

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45. B.E. Richter and L. Covino, *LC-GC,* **18**, 1068 (2000).
46. B.E. Richter, *J. Chromatogr. A,* **874**, 217 (2000).
47. M. Weichbrodt, W. Vetter, and B. Luckas, *J. AOAC Int.,* **83**, 1334 (2000).
48. A.A. Stolker, 'Current trends and development in sample preparation,' in "Proceedings of the EuroResidue IV Conference, Veldhoven, The Netherlands," pp. 148–158 (2000).
49. G.C. Gerhardt, C.D. Salisbury, and H.M. Campbell, *Food Addit. Contam.,* **12**, 731 (1995).
50. L.D. Payne, V.R. Mayo, L.A. Morneweck, M.B. Hicks, and T.A. Wehner, *J. Agric. Food Chem.,* **45**, 3501 (1997).
51. T.D. Herbert, C. Vue, J.A. Bernardy, D.L. Van Horsen, and M.I. Rossulek, *J. AOAC Int.,* **84**, 392 (2001).
52. S.M. Young, D.J. Phillips, P.C. Iraneta, and J. Krol, *J. AOAC Int.,* **84**, 556 (2001).
53. S. Paterson, R. Cordero, S. McCulloch, and P. Houldsworth, *Ann. Clin. Biochem.,* **37**, 690 (2000).
54. S.A. Barker and A.R. Long, *J. Liq. Chromatogr.,* **15**, 2071 (1992).
55. S.E. Katz and M. Siewierski, *J. Chromatogr.,* **624**, 403 (1992).
56. N. Furusawa, *Talanta,* **49**, 461 (1999).
57. N. Furusawa, *J. Liq. Chromatogr. Relat. Technol.,* **23**, 1413 (2000).
58. T. Agasoester, *Food Addit. Contam.,* **9**, 615 (1992).
59. M. Krogh and K.E. Rasmussen, *Methodol. Surv. Bioanal. Drugs,* **23**, 237 (1994).
60. C.E. Tsai and F. Kondo, *J. Liq. Chromatogr.,* **18**, 965 (1995).
61. L.R. Snyder, J.C. Glajch, and J.J. Kirkland, 'Practical HPLC Method Development, second edition, Wiley–Interscience, New York (1997).
62. R.J. Vervoort, F.A. Maris, and H. Hindriks, *J. Chromatogr.,* **623**, 207 (1992).
63. J.J. Kirkland, J.W. Henderson, J.D. Martosella, B.A. Bidlingmeyer, J. Vasta-Russell, and J.B. Adams, *LC-GC,* **17**, 634 (1999).
64. J.J. Kirkland, *J. Chromatogr. Sci.,* **34**, 309 (1996).
65. T. Walter, B. Alden, E. Bouvier, R. Crowley, R. Fisk, C. Gendreau, Z. Jiang, J. O'Gara, and D. Walsh, 'Advances in silica technology for reverse-phase HPLC packings,' Presented at 23rd International Symposium on High Performance Liquid Phase Separation and Related Techniques (HPLC 1999), Granada, Spain, May 30–June 4, 1999.
66. R.E. Hornish and J.R. Weist, *J. Chromatogr. A,* **812**, 123 (1998).
67. B.A. Bidlingmeyer and J.L. Ekmanis, *LC-GC,* **8**, 352 (1990).
68. S. Porter and N. Johnston, 'The application of narrow-bore HPLC columns to the analysis of veterinary drugs,' in "Proceedings of the EuroResidue II Conference," pp. 538–542 (1993).
69. P. Debord, K. Louchahi, and M. Tool, *Fundam. Clin. Pharmacol.,* **7**, 435 (1993).
70. G. Stoev, *J. Chromatogr.,* **710**, 234 (1998).
71. M.P. Turberg, T.D. Macy, J.L. Lewis, and M.R. Coleman, *J. AOAC Int.,* **78**, 1394 (1995).
72. M.S. Ahmed, R.H. Dobberstein, and N.R. Farnsworth, *J. Chromatogr.,* **192**, 387 (1980).
73. S. Einarsson, S. Folestad, B. Josefsson, and S. Lagerkuist, *Anal. Chem.,* **58**, 1638 (1986).
74. R. Willoughby, E. Sheeham, and S. Mitrovich, 'A Global View of LC/MS,' Global View Publishing, Pittsburgh, PA (1998).
75. S.J. Gaskell, *J. Mass Spectrom.,* **32**, 677 (1997).
76. D.G. Kennedy, R.J. McCracken, A. Cannavan, and S.A. Hewitt, *J. Chromatogr. A,* **812**, 77 (1998).
77. P. Furst, 'LC-MS: a powerful tool in residue analysis of veterinary drugs,' in "Proceedings of the EuroResidue IV Conference, Veldhoven, The Netherlands," pp. 63–72 (2000).
78. H. Oka, Y. Ikai, Y. Ito, J. Hayakawa, K. Harada, M. Suzuki, H. Odani, and K. Maeda, *J. Chromatogr. B,* **693**, 337 (1997).
79. J.M. Ballard, L.D. Payne, R.S. Egan, T.A. Wehner, G.S. Rahn, and T. Samson, *J. Agric. Food Chem.,* **45**, 3507 (1997).
80. J. Schloesser, A. Mehlich, F. Ballwanz, and M. Petz, *Anal. Chem.,* **360**, 498 (1998).
81. D.E. Kiehl and A.S. Kennington, *Rapid Commun. Mass Spectrom.,* **9**, 1297 (1995).
82. B.K. Matuszewski, M.L. Constanzer, and C.M. Chavez-Eng, *Anal. Chem.,* **70**, 882 (1998).
83. B.J. Gudzinowicz, 'Gas Chromatographic Analysis of Drugs and Pesticides,' Marcel Dekker, New York, Vol. 2 (1998).
84. A. Braithwaite and F.J. Smith, 'Chromatographic Methods,' fifth edition, Chapman & Hall, London (1996).

85. W. Luo, A. Binzhi, Y.W. Catharina, L. Rushing, and H.C. Thompson, *J. Chromatogr. B,* **687**, 405 (1996).
86. J.E. Matusik, R.S. Sternal, C.J. Barnes, and J.A. Sphon, *J. Assoc. Off. Anal. Chem.,* **73**, 529 (1990).
87. N. Haagsma and C. van der Water, 'Immunochemical methods in the analysis of veterinary drug residues,' "in Analysis of Antibiotic Drug Residues in Food Products of Animal Origin," ed. V. K. Agarwal, Plenum Press, New York, pp. 81–97 (1992).
88. L.D. Rowe, R.C. Beier, M.H. Elissalde, and L.H. Stanker, *J. Agric. Food Chem.,* **42**, 1132 (1994).
89. M.T. Muldoon, C.K. Holtzapple, S.S. Deshpande, R.C. Beier, and L.H. Stanker, *J. Agric. Food Chem.,* **48**, 537 (2000).
90. J.M. Mitchell, A.J. Yee, W.B. McNab, M.W. Griffith, and S.A. McEwen, *J. AOAC Int.,* **82**, 79 (1999).
91. A.G. Baxter, M.C. O'Connor, S.A. Haughey, S.R. Crooks, and C.T. Elliot, *Analyst,* **124**, 1315 (1999).
92. S.L. Weilin and D.J. Smith, *Food Addit. Contam.,* **17**, 837 (2000).
93. R.C. Beier, T.J. Dutko, S.A. Buckley, M.T. Muldoon, C.K. Holtzapple, and L.H. Stanker, *J. Agric. Food Chem.,* **46**, 1049 (1998).
94. D.L. Young, C.A. Mihaliak, S.D. West, K.A. Hanselman, R.A. Collins, A.M. Phillips, and C.K. Robb, *J. Agric. Food Chem.,* **48**, 5146 (2000).
95. US FDA, Guideline for Approval of a Method of Analysis for Residues, US Department of Health and Human Services, Public Health Service, Food and Drug Adminstration, Center for Veterinary Medicine, Washington, DC (2001). Also available on the World Wide Web: **http://www.fda.gov/cvm/guidance/guideline3pt5.html**.
96. US EPA, Residue Chemistry Test Guideline. OPPTS 860.1340 Residue Analytical Chemistry,' US Environmental Protection Agency, Washington, DC (1996). Also available on the World Wide Web: **http://fedbss.access.gpo.gov**.
97. 'Development and Validation of a Proposed Regulatory Method,' EMEA/CVMP/573100, Vol. 8, Veterinary Medicinal Products Establishment of Maximum Residue Limits (MRLs) for Residues of Veterinary Products in Foodstuffs of Animal Origin, Committee for Veterinary Medicinal Products, London (2001). Also available on the World Wide Web: **http://www.emea.eu.int/pdfs/vet/swp/057300en.pdf**.
98. J.A. Sphon, *J. AOAC Int.,* **72**, 1247 (1978).
99. M.R. Coleman, J.W. Moran, and D.H. Mowrey, *J. AOAC Int.,* **80**, 693 (1997).

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Compound class

Anilides

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1 Introduction

Anilides are roughly divided into two main groups of agrochemicals: acylanilides and chloracetanilides. Acylanilides compounds comprise *N*-acylated phenyl ring-substituted anilides with no other substituent on the nitrogen atom, except for mefenacet, which has a methyl group on the nitrogen atom. There are eight representative acylanilide herbicides: chlomeprop, naproanilide, propanil, pentanochlor, etobenzanid, diflufenican, inabenfide and mefenacet. Three representative fungicides are flusulfamide, flutolanil and mepronil. The structures of these compounds and their physico-chemical properties are shown in Figure 1 and Table 1.

Acylanilides (abbreviated as anilides in this article) are generally used as selective pre- and/or post-emergence herbicides in paddy rice fields. The herbicidal activity of the anilides is similar to those exhibited by the auxin-like plant growth regulators.

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2 Residue analytical methods for plant materials

Residue analytical methods for anilides in plant, soil and water samples have been developed. The basic principle consists of the following steps for plants: extraction of the homogenized samples with acetone or other organic solvents and cleanup using liquid–liquid partitioning or column chromatography. Quantitative analyses are carried out either by gas chromatography/electron capture detection (GC/ECD) or gas chromatography/nitrogen–phosphorus detection (GC/NPD) and/or high-performance liquid chromatography/ultraviolet detection (HPLC/UV) or high-performance liquid chromatography/fluorescence detection (HPLC/FL). Column cleanup simplifies the analytical procedure and improves the accuracy and sensitivity of the residue method.

Analytical methods for representative anilides are reported in this article. In addition, they are also applicable as multi-residue methods.

2.1 Nature of the residue

Anilide herbicides are metabolized in plants via cleavage of acylamide moiety.

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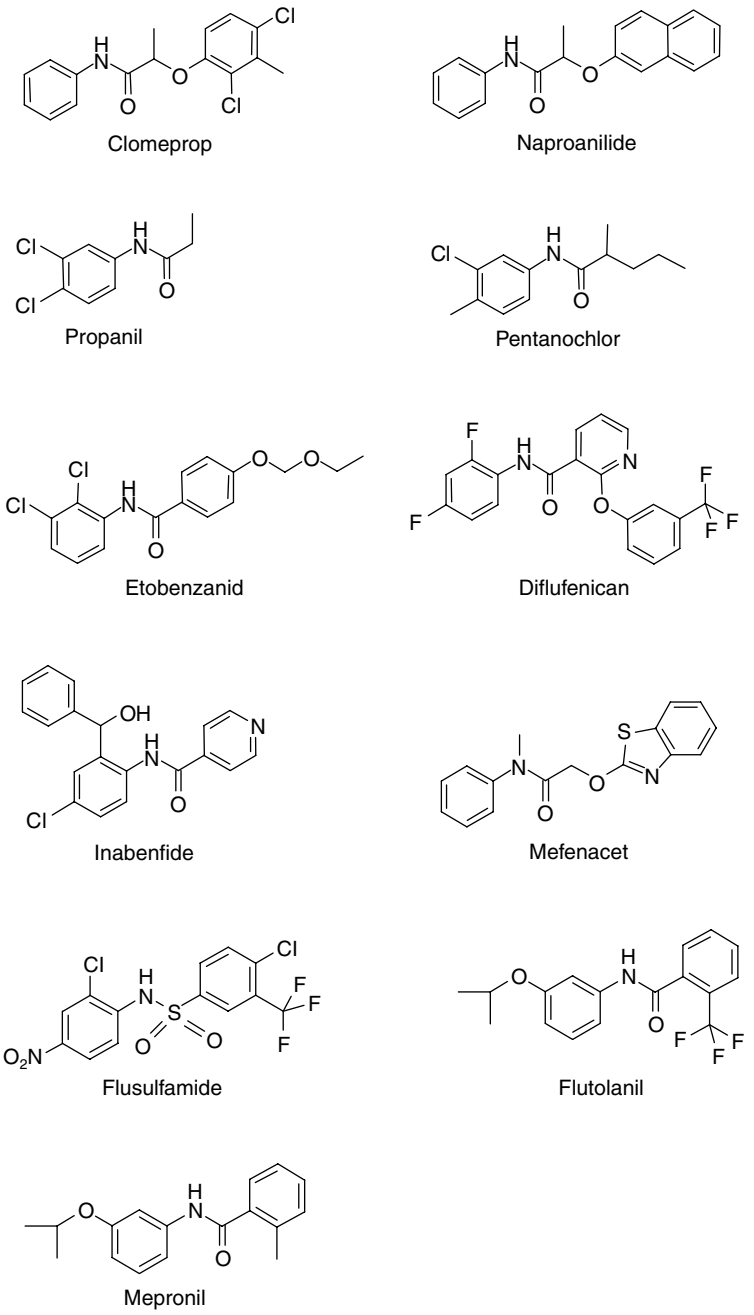


Figure 1 Structures of anilide herbicides and fungicides

The definition of residues for selective anilides in plant samples is summarized below.[1–12]

Compound	*Definition of plant residues*
Clomeprop	Parent and 2-(2,4-dichloro-3-methylphenoxy)propionic acid
Naproanilide	Parent, 2-(2-naphthoxy)propionic acid and methyl 2-(2-naphthoxy)propionate
Propanil	Parent, 3,4-dichloroaniline and propionic acid
Pentanochlor	Parent and 3-chloro-4-methylaniline
Etobenzanid	Parent
Diflufenican	Parent
Inabenfide	Parent
Mefenacet	Parent
Flusulfamide	Parent
Flutolanil	Parent
Mepronil	Parent

2.2 *Analytical method*

The basic principle of the analytical method for anilides is as follows. Homogenized samples such as fruits and vegetables are extracted with acetone or acetonitrile. In the case of brown rice, samples are added to water and allowed to stand for 2 h prior

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Table 1 Physico-chemical properties of anilides

	Vapor pressure (mPa)	Log P_{ow} (20 °C)	Water solubility (mg L^{-1})	Stability		
				Heat	Light	Hydrolysis
Herbicides						
Clomeprop	$<1.3 \times 10^{-2}$ (30 °C)	4.8	0.032 (25 °C)	Stable		Stable (alkaline)
Naproanilide	2.8×10^{-6} (20 °C) 9×10^{-7} (25 °C)	—	0.75 (27 °C)	Deg.	Deg. (in water) Stable (in solid)	Deg. (strongly acidic or alkaline)
Propanil	2×10^{-2} (20 °C) 5×10^{-2} (25 °C)	3.3	130 (20 °C)	—	Deg. (in water)	Stable (pH 4, 7, 9) Deg. (strongly acidic or alkaline)
Pentanochlor	2×10^{-3} (20 °C)	3.6	8–9 (20 °C)	—	Deg.	Stable (pH 7–9)
Etobenzanid	2.1×10^{-2} (40 °C)	4.3	0.92 (25 °C)	Stable (r.t.)		Stable (alkaline) Deg. (acidic)
Diflufenican	4.25×10^{-3} (25 °C)	4.9	<0.05 (25 °C)	Stable	Stable	Stable
Inabenfide	6.3×10^{-2} (20 °C)	3.1	1 (30 °C)	Stable	Stable	Deg. (alkaline)
Mefenacet	6.4×10^{-4} (20 °C)	3.2	4 (20 °C)	Stable	Stable	Stable (pH 4–9)
Fungicides						
Flusulfamide	9.9×10^{-7} (40 °C)	2.8	2.9 (25 °C)	Stable (80 °C)	Stable	Stable
Flutolanil	6.5×10^{-3} (20 °C)	3.7	6.53 (20 °C)	Stable	Stable	Stable (pH 3–11)
Mepronil	5.6×10^{-2} (20 °C)	3.7	12.7 (20 °C)	Stable	Stable	Stable

to extraction with an organic solvent. After evaporation of acetone in the extract, the aqueous residue is transferred into a macroporous diatomaceous column such as a Chem Elut column or partitioned into a nonpolar solvent such as n-hexane. The eluate from the columns or partition solution is evaporated to dryness in vacuum, the residue is dissolved in n-hexane and the solution is subjected to a cleanup procedure using a Florisil or silica gel cartridge, etc. The eluate is evaporated to dryness and analyzed by gas chromatography (GC) or liquid chromatography (LC) using GC/ECD, GC/NPD and HPLC/UV. By this method, clomeprop, propanil, etobenzanide, diflufenican, flusulfamide, flutolanil and mepronil are quantitatively determined. Inabenfide has low vapor pressure, and is difficult to determine directly using GC. The metabolite of clomeprop is also not directly determined by GC, and is derivatized before GC determination as described at Section 2.2.4.

2.2.1 Preparation of analytical samples

A 0.5–2-kg crop sample is cut into small pieces and homogenized thoroughly using a food processor. Rice grain is milled with an ultracentrifuge mill and sieved through a 42-mesh screen. The typical size of an analytical sample is less than 50 g. To prevent the decomposition of the anilide residues, crop samples should be frozen soon after collection and maintained frozen until analyzed.

2.2.2 Extraction

A 10-g sample of the homogenized dry sample is soaked in 20 mL of distilled water for 2 h. After adding 100 mL of acetone to the soaked sample and shaking vigorously on a mechanical shaker for 30 min, the extract is filtered. After the addition of a further 100 mL of acetone, the sample homogenate is shaken as before and the acetone extract is filtered. The filtrates are combined and acetone is removed with a rotary evaporator.[13,14]

2.2.3 Cleanup procedure

The extent of cleanup needed depends on the target analyte, the quality of the sample extract, the method of detection and sensitivity. Liquid–liquid partition (LLP) and solid-phase extraction (SPE) columns such as the C_{18} cartridge and macroporous diatomaceous column are the cleanup method of choice.

(1) LLP

(a) Organic solvent transfer

For the high-performance liquid chromatography (HPLC) determination of naproanilide and its metabolite, 200 mL of 2% sodium sulfate in 0.1 M potassium hydroxide solution are added to the concentrate derived from Section 2.2.2. The solution is shaken twice with 100 mL each of dichloromethane or ethyl acetate–n-hexane (1 : 1, v/v) for 10 min. The combined organic layer is concentrated.[14]

(b) Acetonitrile–n-hexane partition

Acetonitrile–n-hexane partition is an effective method for oily samples, e.g. brown rice (rice bran contains oily materials) and nuts. The residue of organic layer derived from Section 2.2.3(a) is dissolved in 30 mL of n-hexane and naproanilide and its metabolite are extracted twice with 30 mL of acetonitrile. The combined acetonitrile extract is concentrated prior to further cleanup, if needed. This method can be applied to the determination of all other anilide compounds.

(2) Column chromatography

(a) Macroporous diatomaceous column (e.g. Chem Elut column)

The liquid–liquid partition procedure described above can be substituted by using a Chem Elut column. After concentrating the extract derived from Section 2.2 to 20 mL, the concentrate is applied to a Chem Elut column and charged at room temperature for 5–10 min. Naproanilide, propanil and mefenacet are eluted with 80 mL of n-hexane when using the Chem Elut column. The recoveries are in the range 96–110% (personal data).

(b) SPE column: silica gel and Florisil cartridge

The residue of the acetonitrile extract described above is dissolved in 5 mL of n-hexane and applied to a silica cartridge. Naproanilide and its metabolite are eluted with 10 mL of n-hexane–diethyl ether–acetic acid (85 : 15 : 1, v/v/v) after washing the cartridge with 5 mL of n-hexane–diethyl ether–acetic acid (95 : 5 : 1, v/v/v). When using a Florisil column (10-g), naproanilide is eluted with 100 mL of diethyl ether–n-hexane (1 : 1, v/v) after washing the Florisil column with 100 mL of diethyl ether–n-hexane (3 : 17, v/v).[14,15]

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Using a Florisil cartridge, propanil is effectively eluted with 35 mL of diethyl ether–n-hexane (3 : 7, v/v) after rinsing the cartridge with 20 mL of diethyl ether–n-hexane (3 : 17, v/v).

2.2.4 Derivatization

Since the metabolite of clomeprop, 2-(2,4-dichloro-3-methylphenoxy)propionic acid, and inabenfide could not be directly determined by GC, they are derivatized to a more stable compound with diazomethane for the metabolite of clomeprop or with anhydrous chloroacetic acid for inabenfide.

For example, the metabolite of clomeprop is determined by the following procedures. Water (20 mL) is added to 10 g of sample and then the mixture is allowed to stand at room temperature for 2 h. To the mixture, 4 mL of 1 N hydrochloric acid and 100 mL of acetone are added and shaken for 30 min. The filtrate is extracted twice with 50 mL of dichloromethane after 50 mL of water and 5 g of sodium chloride have been added. The combined dichloromethane extract is concentrated, the final residue is dissolved in 50 mL of dichloromethane, then the dichloromethane layer is rinsed with alkaline and acid and extracted twice with 50 mL of 1% sodium bicarbonate–5% sodium chloride (1 : 1, v/v) for 5 min. To the combined aqueous layer, 6 N hydrochloric acid is added and extracted twice with 50 mL of dichloromethane for 5 min. The organic extract is concentrated after drying with anhydrous sodium

sulfate. The residue is treated with 5 mL of diazomethane in diethyl ether at room temperature for 1 h. The reaction mixture is concentrated and the residue is dissolved in 5 mL of n-hexane and applied to a Florisil column (10-g). The methyl derivative of the clomeprop metabolite is eluted with 80 mL of ethyl acetate–n-hexane (3 : 97, v/v) after washing the column with 50 mL of ethyl acetate–n-hexane (1 : 99, v/v). After concentration, the dried eluate is dissolved in 5 mL of n-hexane and cleaned up using a silica gel column (5-g) with the same elution solvent as used in the Florisil column procedure. The eluate is concentrated and the residue is dissolved in a suitable volume of n-hexane and injected into the GC/ECD system. This method is applicable to the determination of other acidic metabolites of anilide compounds. When using a silica cartridge, the metabolite of clomeprop (without derivatization) is eluted with 30 mL of acetone–methanol (1 : 1, v/v) after clomeprop has been eluted with 30 mL of n-hexane–dichloromethane (3 : 2, v/v).

2.2.5 Determination

Several methods can be used for the residue analysis of anilides, especially gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). GC/ECD or GC/NPD for the determination of anilides has generally been used except for the unstable metabolites of naproanilide and clomeprop, which are determined by HPLC/UV, HPLC/FL or GC/ECD after derivatization.

Typical operating conditions by GC and HPLC are listed in Tables 2 and 3, respectively. Anilides are separated using a weakly polar liquid-phase capillary column, such as SPB-1 or HP-5, which is prepared based on 5% diphenyl–95% dimethylpolysiloxane for GC. For HPLC, ODS columns are used.

2.2.6 Evaluation

Quantitation is performed by the calibration technique. A new calibration curve with anilide standard solutions is constructed for each set of analyses. The peak area or peak height is plotted against the injected amount of anilide. The injection volume (2 μL) should be kept constant as the peak area or peak height varies with the injection volume. Before each set of measurements, the GC or HPLC system should be calibrated by injection of standard solutions containing about 0.05–2 ng of anilide. Recommendation: after constructing the calibration curve in advance, standard solutions and sample solutions are injected alternately for measurement of actual samples.

2.2.7 Calculation of residues

The amount of anilide residue (R, mg kg^{-1}) in the sample is calculated by the following equation:

$$R = (W_i / V_i) \times (V_f / G)$$

where

G = sample weight (g)
V_i = injection volume into gas chromatograph or high-performance liquid chromatograph (μL)

Table 2 GC and GC/MS operating conditions for the determination of anilides

	Clomeprop	Clomeprop	Diflufenican	Inabenfide	Mefenacet	Flutoranil	Mepronil	Multi-analysis
Analyte	Clomeprop	Metabolite[a] (derivative)	Diflufenican	Inabenfide	Mefenacet	Flutoranil	Mepronil	
Detection[b]	ECD	ECD	ECD	ECD	NPD	NPD	NPD	MS
Column:	Ultra Bond 20M	OV-225	OV-101	SPB-1	DB-17	DB-5	DB-17	HP-5
i.d. (mm)	2	2	0.53	0.53	0.53	0.53	0.53	0.25
Length (m)	1.2	2.1	30	15	10	15	7	30
Film thickness (μm)						1.5	1	0.53
Temperature (°C):								
Column oven	200	200	230–250	220	240	180, 10 °C min^{-1}, 240	150, 20 °C min^{-1}, 280	100–280
Injection	280	280	250	250	250	250	250	250
Detector	280	280	300	280	280	270	280	280
Flow rate of gas (mL min^{-1}):								
Carrier gas (N_2)	30	30		60				
Carrier gas (He)			4		20	10	10	1
Makeup gas (N_2)			37				20	
Hydrogen					5	3	3	3.5
Air					110	80	100	110
Retention time (min)	2	2	—	4	3	5	5.5	16.2 (mepronil) 18.4 (mefenacet) 18.6 (naproanilide) 12.3 (propanil)
Reference	13	13	16, 17	15, 18	13, 15	19	15, 19	Personal data

[a] 2-(2,4-Dichloro-3-methylphenoxy)propionic acid (DMPA).
[b] ECD, electron capture detection; NPD, nitrogen–phosphorus detection; MS, mass spectrometry.

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Table 3 HPLC operating conditions for the determination of anilides

	Naproanilide	Inabenfide	Etobenzanid	Propanil	Clomeprop	Flusulfamid
Analyte	Naproanilide metabolite[a]	Inabenfide	Etobenzanid	Propanil metabolite[b]	Clomeprop metabolite[c]	Flusulfamid
Detection[d]	FL	UV	UV	PAD	UV	UV
Wavelength (nm)	280–340	280	267	220	234	282
Column:	Pak C_{18}	L-Column	YMC-ODS	U5ODS-30	Inertsil C_8	HP-5
i.d. (mm)	4.6	4.6	6	4.6	4.6	4.6
Length (cm)	25	25	25	15	25	25
Particle size (μm)	5	5	5	5	5	5
Temperature (°C):						
Column oven	40	40	40	40	40	40
Mobile phase (v/v)[e]	MeOH–H_2O–HCOOH (65 : 36 : 0.5)	MeOH–H_2O (7 : 3)	ACN–H_2O (6 : 4)	ACN–H_2O (1 : 19)–(100 : 0) (+0.1% AcOH)	MeOH–acetate buffer (3 : 1)	ACN–H_2O–H_3PO_4 (700 : 300 : 1)
Flow rate of mobile phase (mL min^{-1})	1	1	1	1	0.75	0.8
Retention time (min)[f]	18 (p) 9 (met)	7	25	20.6 (p) 20.5 (met)	21.3 (p) 10.9 (met)	10
Reference	14	18	19	20–23	24	19

[a] 2-(2-Naphthoxy)propionic acid.
[b] 3,4-Dichloroaniline.
[c] 2-(2,4-Dichloro-3-methylphenoxy)propionic acid (DMPA).
[d] FL, Fluorescence; UV, ultraviolet; PAD, photodiode-array detection.
[e] ACN, acetonitrile; HCOOH formic acid; MeOH methanol.
[f] p, parent; met, metabolite.

V_f = final sample volume (mL)
W_i = amount of anilide herbicides for V_i read from calibration curve (ng)

2.2.8 Recoveries, limit of detection and limit of quantitation

The limit of detection (LOD) is an important criterion of the efficiency of an analytical method. It is characterized by the smallest value of the concentration of a compound in the analytical sample. The detectable amount of anilide compounds is in the range 0.01–0.5 ng by GC and 0.1–4 ng by HPLC. The limit of quantitation (LOQ) ranges from 0.005 to 0.01 mg kg^{-1} for vegetables, fruits and crops. The recoveries from untreated plant matrices with fortification levels between 10 and 50 times the LOD and the LOQ are 70–120%. The relative standard deviation (RSD) at 10–50 times the level of the LOD and LOQ are $\leq$10 % and $\leq$20%, respectively.

Using multi-analytical methods, most of the anilides, including naproanilide, propanil and mefenacet, show recoveries of >70% from 0.5 mg kg^{-1} fortified tomato.[25] According to the official analytical method of the Ministry of Environment, Japan,[13] the recovery of clomeprop fortified at 0.4 mg kg^{-1} in brown rice is >90% (personal data).

2.2.9 Other analytical methods

(1) Mefenacet

Garrido *et al.*[26] developed an electrochemical procedure for the determination of mefenacet residues in rice crops. Optimization of parameters such as pH, frequency and electrochemical electrode surface treatment is necessary to obtain accurate analytical data.

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(2) Inabenfide

Watanabe *et al.*[27] developed an enzyme-linked immunosorbent assay (ELISA) for the detection of inabenfide, a plant growth regulator, in rice. Specific monoclonal antibody (MAB) is used for this method. The effects of rice matrices on the sensitivity of ELISA can be reduced by adding 0.1% Tween 20. Good reproducibility and accuracy of the proposed ELISA were obtained for rice samples and the recovery was 92% at a fortification level of 5–500 μg kg^{-1}.

2.2.10 Multi-residue analysis

Based on the recent rapid progress in multi-residue analytical technology, 100 or more pesticides have been determined by current multi-residue analytical methods. The methods basically consist of SPE and GC/MS or LC/MS. For example, Fillion *et al.*[28] reported a method to determine the residues of 251 pesticides in fruit and vegetable samples. The sample is extracted with acetonitrile, followed by a salting-out step. An aliquot of the acetonitrile extract is passed through a C_{18} cartridge to remove the nonpolar co-extracts. The eluate is applied to a carbon SPE cartridge coupled with an aminopropyl cartridge and eluted with acetonitrile–toluene (3 : 1, v/v). Residual pesticides are determined by GC/MS and HPLC with post-column reaction or fluorescent detection. By this method, the recovery of propanil fortified at 0.1 mg kg^{-1} was 94%.

2.2.11 Important points in analysis

(1) Analytical procedure

Homogenizing and milling for brown rice and rice straw samples must be carried out while freezing with dry-ice. During evaporation of organic solvents, the temperature of the water-bath should be kept at 40 °C or lower.

(2) Peak type on chromatogram

The shape of the matrix peaks depends on the nature of the sample and also on the composition of the HPLC solvent system. For an HPLC column, a low level of detection requires that interfering peaks in the samples be minimal.

3 Residue analytical methods for soil

3.1 Nature of the residues

Clomeprop, naproanilide, propanil and pentanochlor are unstable to sunlight in/on the soil surface. The definition of residues in soil samples is summarized below.

Compound	*Definition of soil residues*
Clomeprop	Parent and 2-(2,4-dichloro-3-methylphenoxy)propionic acid
Naproanilide	Parent and 2-(2-naphthoxy)propionic acid
Propanil	Parent and 3,4-dichloroaniline
Pentanochlor	Parent and 3-chloro-4-methylaniline
Etobenzanid	Parent
Diflufenican	Parent and 2-[3-(trifluoromethyl)phenoxy]-3-pyridinecarboxylic acid
Inabenfide	Parent
Mefenacet	Parent, benzothiazole and benzothiazolyoxyacetic acid
Flusulfamide	Parent
Flutolanil	Parent
Mepronil	Parent

3.2 Analytical method

3.2.1 Preparation of analytical samples

In the laboratory, soil samples collected in the field are mixed thoroughly and reduced in size to laboratory samples. The air-dried soils are passed through a 2-mm sieve in order to remove stones and roots, then the water content of the soil is calculated after drying at 105 °C for 5 h. If the analytical samples cannot be analyzed immediately after drying and sieving, they should be stored at about −20 °C in glass or Teflon bottles fitted with screw-caps.

3.2.2 Extraction

Extraction of residues from soil samples is much more difficult than their extraction from plant or water samples. The pesticide residues in the soil exist often in several forms as 'bound residue', which may affect the extraction efficiency of pesticides from the soil. Then, various extraction methods such as organic solvent extraction, Soxhlet extraction, sonication extraction, microwave dissolution and supercritical fluid extraction (SFE) are used. Some extraction methods are described in the following.

(1) Organic solvent extraction

The analytical method for diflufenican is as follows. A 50-g soil sample is extracted with 100 mL of acetonitrile for 45 min with a rotary shaker at 240 rpm. The mixture is centrifuged for 10 min at 3000 rpm, the supernatant is filtered through a glass filter funnel with anhydrous sodium sulfate and the filtrate is collected.[16]

(2) Soxhlet extraction

Soxhlet extraction followed by liquid chromatography/photodiode-array detection (LC/PAD) is used for the trace determination of propanil and its major metabolite, 3,4-dichloroaniline, in soil. A 10-g soil sample is extracted with methanol in a Soxhlet system for 8 h. After the extracts have been concentrated to dryness, the residue is dissolved in 500 μL of n-hexane.[20]

The other extraction method is an SFE procedure, which is a much easier procedure for extracting the nonpolar chemicals compared with polar chemicals.

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3.2.3 Column cleanup procedure

(1) SPE column: silica gel cartridge

The extract of diflufenican derived from Section 3.2.2(1) is concentrated to dryness and the residue is dissolved in 2 mL of dichloromethane, and 1 mL of this solution is transferred into a silica gel cartridge (2 g) previously conditioned with dichloromethane. Diflufenican is eluted with dichloromethane.

(2) SPE column: Florisil cartridge

Propanil and its metabolite in the n-hexane phase of the soil extract derived from Section 3.2.2(2) are passed through a Florisil column (previously activated at 300 °C overnight and deactivated with 2% water; 2 g). Propanil residues are eluted with 20 mL of diethyl ether–n-hexane (1 : 1, v/v).

The other column chromatography cleanup procedure uses a macroporous diatomaceous column (e.g., Chem Elut column) and an SPE column; C_{18} cartridges are effective columns for sample cleanup.

3.2.4 Determination, evaluation and calculation of residues

The determination, evaluation and calculation of residues procedures are described in Sections 2.2.5–2.2.7.

3.2.5 Recoveries, limit of detection and limit of quantitation

The recoveries of clomeprop and its metabolite DMPA in soil after extraction with acetonitrile–water (4 : 1, v/v) by HPLC/UV (234 nm) are >90%.[24] The recoveries of propanil and its metabolite, 3,4-dichloroaniline, in soil are 95 and 76%, respectively, by Soxhlet extraction and LC/PAD determination.[20] Diflufenican is added to soil in the range 0.002–0.008 mg kg^{-1} to validate the method developed by Conte *et al.*[16] The average recovery from the soil by this method is $92 \pm 5\%$.

3.2.6 Other analytical methods

A sensitive method has been developed to determine the aged residues of diflufenican in soil by GC/ECD. A sample extraction using 100% methanol with 'extended shake' was performed. The extract was concentrated and purified using a C_{18} SPE column. Further cleanup was effected by using a silica SPE column. The LOD for diflufenican in soil was 0.001 mg kg^{-1}. The recovery of diflufenican at fortification levels from 0.02 to 0.2 mg kg^{-1} in soil by this method was between 94 and 121%.[17]

Zhu *et al.*[29] developed a pressurized fluid extraction (PFE) for the quantitative recovery of chloroacetanilide and nitrogen-containing heterocyclic herbicides in soil, based on elevated temperatures and pressures using liquid solvents. PFE (with water) is more effective than SFE, which requires organic and inorganic modifiers for the extraction of intermediates of polar compounds. For example, chloroacetanilide in soil was completely recovered by water-assisted PFE. The sample was loaded into the extraction vessel, which was filled with extraction solvent by opening the pump valve. An air-dried soil sample (5-g) was weighed into a 10-mL beaker. The water content of the soil sample was adjusted to 38%. The sample was mixed well and covered with aluminum foil. After the sample had been incubated for 1 h, it was transferred to an 11-mL stainless-steel extraction cell, which was prefilled with solvent. The cell containing the sample was placed in a 5-mL oven for 5-min static extraction at 1500 psi extraction pressure and 100 °C extraction temperature. Average recoveries of the tested pesticides ranged from 93 to 103% with the water-assisted PFE method, compared with only 68–83% when no water was used.

This method may also be applicable to acylanilides herbicides.

3.2.7 Important points

(1) Extraction

Centrifuging the screw-cap vial can easily break emulsions, which often form during extraction. The vial will survive up to 6000 g if rubber stoppers are inserted into the centrifuge cup to provide a flat base for the vials. The required phase (usually the top layer) can be easily removed with a pipet or, if it is to be discarded, it can be removed using a disposable pipet connected by tubing to a suction flask and vacuum line.

(2) High organic content soils

A multi-residue method based on SPE cleanup and gas chromatography/ion trap mass spectrometry (GC/ITMS) was developed for the determination of 120 pesticides and related metabolites in two soils with organic matter contents of 4.0–5.2%.

The recoveries of propanil in soils containing 5.2% organic matter and 4.0% organic matter were 101 and 96%, respectively, with an LOD of <1 mg kg^{-1}.[30]

4 Analytical methodology for water

4.1 Nature of the residues

Most of the anilides except for naproanilide, propanil and pentanochlor are stable to sunlight in water. The definitions of residues in water samples are summarized below.

Compound	*Definition of water residues*
Clomeprop	Parent and 2-(2,4-dichloro-3-methylphenoxy)propionic acid
Naproanilide	Parent and 2-(2-naphthoxy)propionic acid
Propanil	Parent and 3,4-dichloroaniline
Pentanochlor	Parent and 3-chloro-*p*-toluidine
Etobenzanid	Parent and 4-ethoxymethoxybenzoic acid
Diflufenican	Parent
Inabenfide	Parent
Mefenacet	Parent
Flusulfamide	Parent
Flutolanil	Parent
Mepronil	Parent

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4.2 Analytical method

4.2.1 Sample preparation

Samples generally should be prepared or at least extracted immediately after collection or after arrival in the laboratory. If it is not possible for water samples to be prepared or extracted immediately, they should be stored at 5 °C until analysis, in order that no transformation or degradation products occur.

4.2.2 Extraction

Several extraction methods for water samples are applicable, such as solvent extraction, SPE using a cartridge and disk and solid-phase microextraction (SPME) .

(1) Organic solvent extraction

Almost all anilides in water samples are directly extracted with ethyl acetate or dichloromethane, and the method of multi-residue analysis can be applied to the water samples. However, in the case of naproanilide, the water sample is extracted with an organic solvent under acidic conditions. A 5-mL volume of 1 N hydrochloric acid and 50 mL of ethyl acetate–n-hexane (1 : 1, v/v) are added to 200 mL of water sample, and the mixed solution is shaken vigorously using a mechanical shaker for

5 min at room temperature. The organic layer is separated and the aqueous layer is extracted again with 50 mL of the same solvent mixture. The combined organic layer is dehydrated with anhydrous sodium sulfate and concentrated.[13]

To determine simultaneously the parent compounds and the metabolites of ethobenzanid and clomeprop, each parent compound is extracted with n-hexane from the water sample, and the metabolites are extracted with diethyl ether after acidification of the remaining aqueous layer.

(2) SPE column: C_{18} cartridge

An SPE method has been developed to replace the classical LLP method. Water sample is extracted with an SPE column such as C_{18} and styrene–divinylbenzene copolymer (PS-2) cartridges, which consist of a reversed bonded-phase silica sorbent, provided as an extraction tool. This is a simple and rapid method, and applied to the determination of residual amounts of naproanilide, propanil, mefenacet, etc.[31–34] This system determines the residual amounts of most of the pesticides and has been successfully applied to determination of pesticides in water.

The official analytical method of the Ministry of Environment, Japan,[13] recommends the use of a C_{18} cartridge to determine naproanilide. This method consists of the following procedure: 5 mL of the 1 N hydrochloric acid are added to the water sample and the solution is applied to a C_{18} cartridge, which is preconditioned with 5 mL each of acetonitrile and water, at a flow rate of 10–20 mL min^{-1}. Naproanilide and its metabolite, 2-(2-naphthoxy)propionic acid, are eluted with 10 mL of acetonitrile.

A monitoring system has been established to determine 90 pesticides including anilides and 10 related degradation products in river water. Pesticide residues in the water sample are collected on a PS-2 cartridge (265-mg) at a flow rate of 10 mL min^{-1}, eluted with 3 mL of acetone, 3 mL of n-hexane and 3 mL of ethyl acetate successively, and determined by GC/MS. Overall recoveries ranged from 72 to 118%. Recoveries of mepronil, naproanilide, propanil and flutolanil at fortification levels of 0.1 and 2 mg kg^{-1} in water by this method were 80–112%. The LODs were 0.01–0.1 μg L^{-1}.[33]

Thus, organic solvent extraction methods for the extraction of pesticides from water samples can be replaced by the SPE method using C_{18} and PS-2. Ethobenzanid, clomeprop, naproanilide and their acidic metabolites are determined by a multi-residue analytical method using C_{18} or PS-2 cartridge extraction after acidification of the water samples with hydrochloric acid or other acidic media, followed by HPLC or LC/MS detection.

(3) SPE disk

A multi-residue method for 25 selected pesticides including propanil using an SPE disk has also been developed as a rapid screening method for organic contaminants in river, lake and seawater samples. C_{18} SPE disks are conditioned with 10 mL of acetone for 3 h. Water samples (1 L) are allowed to percolate through the disks in order to trap the residues at a flow rate of 50 mL min^{-1} under vacuum. Residues trapped in the disks are extracted twice by eluting with 5 mL of dichloromethane–ethyl acetate (1 : 1, v/v). The more hydrophobic compounds ($\log K_{ow} > 3$) seem to show no

significant decrease in their recoveries by this method from lake and gulf water owing to the higher organic carbon content.[35,36]

(4) SPME

A procedure involving SPME followed by GC/MS has been developed to determine propanil in water samples. A Carbowax–divinylbenzene SPME fiber is used. Linearity of the calibration curves is attained in the range 0.1–10 $\mu g\,L^{-1}$ in water samples.[23]

4.2.3 Determination, evaluation and calculation of residues

The determination procedure, evaluation and calculation of residues are described in Sections 2.2.5–2.2.7.

4.2.4 Recoveries, limit of detection and limit of quantitation

GC (GC/MS) or HPLC (LC/MS) determination after SPE extraction is carried out as the standard method for most anilides. Ninety pesticides including anilides show recoveries ranging from 72 to 118% with GC/MS. The LOD is 0.01–0.1 $\mu g\,L^{-1}$.[33]

An automated on-line SPE procedure followed by LC/PAD has been investigated to determine different classes of pesticides including propanil in water samples containing various amounts of humic substances. Good recoveries were obtained at neutral pH for most of the analytes up to 40 $mg\,L^{-1}$ of humic acid, but were significantly affected at both acidic and neutral pH only at 80 $mg\,L^{-1}$. The LODs obtained for water containing 10 $mg\,L^{-1}$ of humic acid were between 0.05 and 0.3 $ng\,mL^{-1}$.[20]

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4.2.5 Other analytical methods

A rapid homogeneous polarization fluorescence immunoassay (PFIA) has been developed for propanil. The method can detect propanil concentrations in the range 1–100 $ng\,mL^{-1}$ in 50 mL of sample solution. The analytical process for one sample takes less than 1 min. In order to achieve better sensitivity, a preconcentration step is a possible process and has been applied for samples with low concentrations. The use of SPE is suitable for enriching the analytes in the sample, allowing the water samples to be measured at the LOD levels required. Both SPE followed by PFIA and SPME followed by GC/MS generate consistent analytical data when propanil is determined in drinking water.[37]

Crescenzi *et al.*[38] developed a multi-residue method for pesticides including propanil in drinking water, river water and groundwater based on SPE and LC/MS detection. The recoveries of the pesticides by this method were $>80\%$. Santos *et al.*[22] developed an on-line SPE method followed by LC/PAD and LC/MS detection in a simultaneous method for anilides and two degradation products (4-chloro-2-methylphenol and 2,4-dichlorophenol) of acidic herbicides in estuarine water samples. To determine the major degradation product of propanil, 3,4-dichloroaniline, the positive ion mode is needed for atmospheric pressure chemical ionization mass spectrometry (APCI/MS) detection. The LOD of 3,4-dichloroaniline by APCI/MS was 0.1–0.02 $ng\,mL^{-1}$ for 50-mL water samples.

References

1. M. Oyamada and S. Kuwatsuka, *J. Pestici. Sci.,* **11**, 179 (1986).
2. K. Kobayashi and K. Ichinose, *Weed Res. Jpn.,* **32**, 46 (1987).
3. G.G. Still, *Plant Physiol.,* **43**, 543 (1968).
4. R.Y. Yih, D.H. McRae and H.F. Wilson, *Science*, **161**, 376 (1968).
5. G.R. Stahler and W. Pestemer, 'Hum. Environ. Expos. Xenobiot. Proceedings of the XI Symposium on Pesticide Chemistry, Cremona', ed. A.A.M. Del Re, C. Brown, E. Capri, G. Errera, S.P. Evans, and M. Trevisan, p. 433 (1999).
6. R. Bartha, *Science*, **166**, 1299 (1969).
7. R. Bartha, *J. Agric. Food Chem.,* **19**, 385 (1971).
8. J. Rouchard, F. Gustin, M.V. Himme, R. Bulcke, F. Benoit, and K. Maddens, *J. Agric. Food Chem.,* **39**, 968 (1991).
9. T. Yoshida and Y. Nakano, *Kagaku Kogaku Ronbunshu*, **26**, 842 (2000).
10. A. Dahchour, G. Bitton, C.M. Coste, and J. Bastide, *Bull. Environ. Contam. Toxicol.,* **36**, 556 (1986).
11. I. Correa and W.C. Steen, *Chemosphere*, **30**, 103 (1995).
12. C.D.S. Tomlin (ed.), 'The Pesticide Manual,' 12th edition, British Crop Protection Council, Farnham, p. 593 (2000).
13. Ministry of Environment, 'The Official Analytical Methods of the Ministry of Environment, Japan,' Ministry of Environment, Tokyo, mefenacet (1993), clomeprop (1996), naproanilide (2000) (in Japanese).
14. S. Goto and S. Kato, 'Analytical Methods for Pesticides,' Soft Science, Tokyo, p. 263 (1987).
15. M. Ueji, H. Kobayashi, and K. Nakamura, 'Analytical Methods for Pesticide Residues,' Soft Science, Tokyo, p. 313 (inabenfide), p. 356 (mefenacet), p. 381 (naproanilide) (2001) (in Japanese).
16. E. Conte, G. Morali, M. Galli, G. Imbroglini, and C. R. Leake, *J. Agric. Food Chem.,* **46**, 4766 (1998).
17. E. Wennberg and L. Torstensson, *Int. J. Environ. Anal. Chem.,* **67**, 73 (1997).
18. K. Sasaki, T. Tasuno, M. Nakamura, A Anazawa, T. Imazawa, O. Usunomiya, S. Terasawa, H. Osada, M. Kaneko, and M. Yoyoda, *J. Food Hyg. Soc. Jpn.,* **42**, 269 (2001).
19. Ministry of Health, Welfare and Labor, 'The Official Analytical Methods of the Ministry of Environment, Japan,' Ministry of Health, Welfare and Labor, Tokyo, mepronil (1993), flutoranil (1996), flusulfamid (1996), etobenzanid (1997) (in Japanese).
20. T.C.R. Santos, L.C. Rocha, and D. Barcelo, *Int. J. Environ. Anal. Chem.,* **70**, 19 (1998).
21. T.C.R. Santos, J.C. Rocha, R.M. Alonso, E. Martinez, C. Ibanes, and D. Barcelo, *Environ. Sci. Technol.,* **32**, 3479 (1998).
22. T.C.R. Santos, J.C. Rocha, and D. Barcel, *J. Chromatogr. A*, **879**, 3 (2000).
23. M. Natangelo, S. Tavazzi, R. Fanelli, and E. Benfenati, *J. Chromatogr. A*, **859**, 193 (1999).
24. K. Kobayashi, Y. Tsukasaki, S. Tongma, and I.S. Shim, *Pestici. Sci.,* **55**, 474 (1999).
25. K. Iijima, M. Saka, Y. Odanaka, and O. Matano, *J. Pestici. Sci.,* **22**, 17 (1997).
26. E.M. Garrido, J.L.C. Lima, C.M. D-Matos, and A.M.O. Brett, *Int. J. Environ. Anal. Chem.,* **75**, 149 (1999).
27. E. Watanabe, Y. Tsuda, S. Watanabe, S. Ito, M. Hayashi, T. Watanabe, Y. Yuasa, and H. Nakazawa, *Anal. Chim. Acta*, **424**, 149 (2000).
28. J. Fillion, F. Sauvé, and, J. Selwyn, *J. AOAC Int.,* **83**, 698 (2000).
29. Y. Zhu, K. Yanagihara, F.M. Guo, and Q.X. Li, *J. Agric. Food Chem.,* **48**, 4097 (2000).
30. P. Mogadati, J.B. Louis, and J.D. Rosen, *J. AOAC Int.,* **82**, 705 (1999).
31. H. Kobayashi, K. Ohyama, N. Tomiyama, Y. Jimbo, O. Matano, and S. Goto, *J. Chromatogr.,* **643**, 197 (1993).
32. Y. Shimamura, N. Tomiyama, M. Murakoshi, H. Kobayashi, and O. Matano, *J. Pestic. Sci.,* **23**, 241 (1998).
33. A. Tanabe, H. Mitobe, K. Kawata, and M. Sakai, *J. AOAC Int.,* **83**, 61 (2000).

34. R. Tauler, D.D. Azevedo, S. Lacorte, R. Cespesed, P. Viana, and D. Barcelo, *Environ. Technol.,* **22**, 1043 (2001).
35. T.A. Albanis and D.G. Hela, *J. Chromatogr. A*, **707**, 283 (1995).
36. T.A. Albanis, D.G. Hela, T. Sakellarides, and I.K. Konstantinou, *J. Chromatogr. A*, **823**, 59 (1998).
37. A.I. Krasnova, S.A. Eremin, M. Natangelo, S. Tavazzi, and E. Benfenati, *Anal. Lett.,* **34**, 2285 (2001).
38. C. Crescenzi, A.D. Corcia, E. Guerriero, and R. Samperi, *Environ. Sci. Technol.,* **31**, 479 (1997).

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Chloroacetanilide herbicides

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1 Introduction

Chloroacetanilides are soil-applied herbicides used for pre- and early post-emergence control of annual grasses and broadleaf weeds in crops. Representative chloroacetanilide compounds, alachlor, acetochlor, and metolachlor, are extensively used worldwide. Other chloroacetanilides with limited usages include propachlor, butachlor, metazachlor, pretilachlor, and thenylchlor. Public environmental concerns and government regulatory requirements continue to prompt the need for reliable methods to determine residues of these herbicides. There now exist a variety of analytical methods to determine residues of these compounds in crops, animal products, soil, and water. The chemical structures and major crops in which these compounds are used are summarized in Table 1.

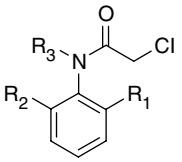
The focus of this article is to describe the residue methodologies for alachlor, acetochlor, metolachlor, and propachlor. Four residue analytical methods are discussed:

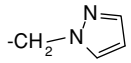
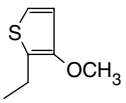
1. method for the determination of acetochlor and its metabolites in plants and animals
2. method for the determination of propachlor and its metabolites in plants and animals
3. multi-residue method for the determination of acetochlor, alachlor, and metolachlor in aqueous samples
4. multi-residue method for the determination of acetochlor, alachlor, and metolachlor and their soil metabolites in aqueous samples.

The current methodology to determine residues of alachlor, acetochlor, propachlor, and butachlor in crops and animal products was developed over the last two decades by researchers at the Monsanto Company. These herbicides degrade rapidly in plants and animals to numerous metabolites that can be hydrolyzed to common aniline moieties. Little to no parent herbicide is found as intact residue in crops and animal products; therefore, the residue methodology focuses on the determination of the common moieties that are derived from the parent herbicides and their metabolites. Initially, gas chromatography (GC) with flame ionization detection, nitrogen–phosphorus

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Table 1 Chloroacetanilides and major crops



R_1	R_2	R_3	Compound	CAS No.	Major crops
Me	Et	$-CH_2OC_2H_5$	Acetochlor	34256-82-1	Maize, soybeans, sunflowers, peanuts
Et	Et	$-CH_2OCH_3$	Alachlor	15972-60-8	Maize, soybeans, sorghum, sugarcane, oilseed crops, peanuts
Et	Et	$-CH_2OC_4H_9$	Butachlor	23184-66-9	Rice
Me	Me		Metazachlor	67129-08-2	Potato, oilseed rape, soybeans, vegetables
Me	Et	$CH(CH_3)CH_2OCH_3$	Metolachlor	51218-45-2	Maize, soybeans, peanuts, sorghum, potato, cotton
Et	Et	$-CH_2CH_2OC_4H_7$	Pretilachlor	51218-49-6	Rice
H	H	$-CH(CH_3)_2$	Propachlor	1918-16-7	Maize, sorghum, vegetables
Me	Me	 attached on end carbon of ethyl moiety	Thenylchlor	96491-05-3	Rice

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detection (NPD), or electron capture detection (ECD) was used to separate and quantify residues. The latter two detection methods are still preferred for the determination of common moieties of propachlor and butachlor. Metabolites of alachlor and acetochlor hydrolyze to anilines that are less volatile and, under certain conditions, thermally unstable. High-performance liquid chromatography (HPLC) with oxidative electrochemical detection (OECD) was developed as the separation/detection method of choice for quantifying the residues derived from alachlor and acetochlor. A gas chromatography/nitrogen–phosphorus detection (GC/NPD) method for propachlor and a high-performance liquid chromatography/oxidation electrochemical detection (HPLC/OECD) method for acetochlor are described in this article as representative methods for the determination of chloroacetanilide residues in plants and animal products.

Analytical methods for parent chloroacetanilide herbicides in soil typically involve extraction of the soil with solvent, followed by solid-phase extraction (SPE), and analysis by gas chromatography/electron capture detection (GC/ECD) or gas chromatography/mass spectrometry (GC/MS).[1] Analytical methods for parent chloroacetanilides in water are similarly based on extraction followed by GC with various detection techniques. Many of the water methods, such as the Environmental Protection Agency (EPA) official methods, are multi-residue methods that include other compound classes in addition to chloroacetanilides.[2–4] While liquid–liquid partitioning was used initially to extract acetanilides from water samples,[5] SPE using

cartridges or disks is currently considered the technique of choice.[6] These methods use nitrogen-sensitive detection,[2,5] ECD,[3] or mass spectrometry (MS) for detection.[4,6,7] MS detection has an advantage over NPD and ECD for both water and soil samples, because MS detection permits confirmation of the identity of the herbicide and/or its fragments and has become more convenient and more affordable in recent years. A GC/MS multi-residue method with internal standards to determine parent herbicides in water is presented in this article as a representative method for the analysis of environmental samples. This method could be adapted for use without internal standards.

The development of analytical methodology for metabolites of acetanilides in soil and water is a particular challenge owing to the presence of numerous analytes and the difficulty in separating many similar metabolites of related compounds. Furthermore, to add to the complexity, acetanilide metabolites can exist as rotational isomers owing to restricted rotation around the amide bond or the bond to the aromatic ring when the ring is asymmetrically substituted.[8] These rotational isomers, owing to restricted rotation around the amide bond, generally inter-convert rapidly but in some cases are separated into two distinct peaks by HPLC. Because the major soil metabolites of acetanilides are polar and nonvolatile, GC cannot be used for analysis unless the analytes are derivatized, which, for some metabolites, particularly the sulfonic acids, is difficult. For this reason, HPLC is typically used for separation.

For controlled study situations where there are no interferences from other agrochemicals, methods for determination of acetanilide metabolites in soil or water can be relatively straightforward. This is not the case for monitoring samples collected in the environment, which can contain a wide array of agrochemicals and metabolites. The analytical methods usually involve extraction of the compound(s) from the matrix, typically using SPE for water,[9,10] followed by HPLC separation. Detection methods for these methods can include ultraviolet (UV), diode-array detection (DAD),[11] MS[12] and tandem mass spectrometry (MS/MS).[13,14] Owing to their similarities in structure and, in some cases, identical molecular weights of parent or fragment ions, liquid chromatography/tandem mass spectrometry (LC/MS/MS) is the best technique to ensure that the identity of each particular analyte in environmental samples is confirmed, and a multi-residue method by direct aqueous injection LC/MS/MS is presented in this article.

Existing immunoassay detection methods for chloroacetanilide herbicides and their metabolites in water or soil are convenient and inexpensive, and they are useful for screening samples either as a group or as individual compounds in the laboratory and in the field. Unfortunately, these methods, while selective for some compound classes, are not reliable enough at sub-parts per billion ($\mu g\,kg^{-1}$ or $\mu g\,L^{-1}$) levels to use for definitive determinations of chloroacetanilides, mainly owing to cross-reactivity with compounds of similar structure, leading to false positives.[15–19] Alternative analytical methods with greater specificity at sub-parts per billion levels, such as those described in this article, are required to confirm positive detections resulting from immunoassay methods.

In the following sections, the nature of chloroacetanilide residues in plants, animal products, water, and soil and the rationale for the analytical methodology that is presented are briefly summarized. Procedures for representative methods are included in detail. The methods presented in this article are among the best available at this time, but analytical technology continues to improve. Future directions for acetanilide residue methodology for environmental monitoring are discussed at the end of the article.

2 Analytical methodology for plant and animal products

2.1 Nature of the residue

Plant and animal metabolism studies have shown that alachlor, acetochlor, propachlor, and butachlor are metabolized to a diverse array of metabolites, the majority of which retain the aniline moiety as their core. The metabolic pathways can involve, to various degrees, such processes as oxidative replacement of chlorine, cleavage of the *N*-alkoxymethyl side chain, and glutathione displacement of chlorine, followed by formation of sulfur-containing degradation products.[20] The parent chloroacetanilides are not found in any crop fraction. For the purposes of residue analysis, plant and animal metabolites of alachlor, acetochlor, and butachlor are generally distinguished as two classes of structures, by the absence or presence of hydroxylation in the alkyl ring substituents.

2.2 Rationale for the presented methods

The complexity of the metabolism of alachlor, acetochlor, butachlor, and propachlor has led to the development of degradation methods capable of hydrolyzing the crop and animal product residues to readily quantitated degradation products. Alachlor and acetochlor metabolites can be hydrolyzed to two major classes of hydrolysis products, one which contains aniline with unsubstituted alkyl groups at the 2- and 6-positions, and the other which contains aniline with hydroxylation in the ring-attached ethyl group. For alachlor and acetochlor, the nonhydroxylated metabolites are hydrolyzed in base to 2,6-diethylaniline (DEA) and 2-ethyl-6-methylaniline (EMA), respectively, and hydroxylated metabolites are hydrolyzed in base to 2-ethyl-6-(1-hydroxyethyl)aniline (HEEA) and 2-(1-hydroxyethyl)-6-methylaniline (HEMA), respectively. Butachlor is metabolized primarily to nonhydroxylated metabolites, which are hydrolyzed to DEA. Propachlor metabolites are hydrolyzed mainly to *N*-isopropylaniline (NIPA). The base hydrolysis products for each parent herbicide are shown in Figure 1. Limited interference studies have been conducted with other herbicides such as metolachlor to confirm that its residues are not hydrolyzed to the EMA under the conditions used to determine acetochlor residues. Nonhydroxylated metabolites of alachlor and butachlor are both hydrolyzed to the same aniline, DEA, but these herbicides are not used on the same crops.

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2.3 Description of methodology

Residues of alachlor and acetochlor are determined by similar methods involving extraction, hydrolysis to the common aniline moieties, and separation and quantitation by reversed-phase HPLC with electrochemical detection. The analytical method for acetochlor is included as a representative method for residue determination of alachlor and acetochlor in plant and animal commodities. Propachlor and butachlor residues, both parent and metabolite, are determined by similar analytical methods involving extraction, hydrolysis to common aniline moieties, and separation and quantitation by capillary GC. The analytical method for propachlor is included as a representative method. The details of the analytical methods for acetochlor and propachlor are presented in Sections 4 and 5, respectively. Confirmation of the residue in a crop or

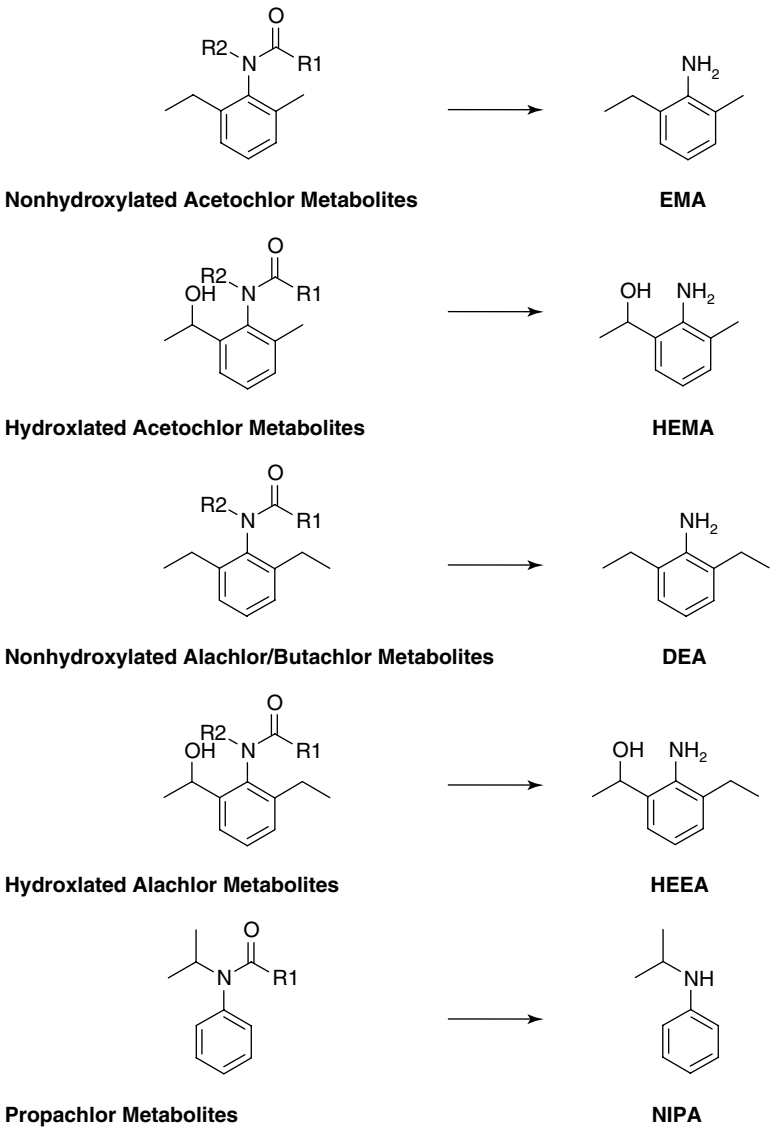


Figure 1 Base hydrolysis products of chloroacetanilide herbicides

animal matrix can be obtained by quantitation of the *N*-perfluoroacylated derivatives of the anilines using GC/ECD by or GC/MS.

3 Analytical methodology for water and soil

3.1 Nature of the residue

Propachlor, alachlor, acetochlor, and butachlor degrade readily and extensively in soil mainly through displacement of chlorine followed by further metabolism to numerous

degradation products. In aerobic soil metabolism studies conducted with alachlor, acetochlor, and propachlor, the most abundant metabolites have been identified as the water-soluble oxanilic, sulfonic, and sulfinyl acetic acids.[20] Parent acetanilide herbicides such as metolachlor, acetochlor, and alachlor and their oxanilate and sulfonate metabolites have been found in surface water and groundwater.[21–23] Structures of these latter oxanilate and sulfonate metabolites are shown in Section 7.

3.2 Rationale for the methods presented

Two multi-residue methods are described. These methods have been used to determine concentrations of either parent herbicides or their metabolites in thousands of surface water and groundwater samples collected over the years 1995–2001 in corn-growing areas of the United States.[21–23]

3.2.1 Parent compounds

The method using GC/MS with selected ion monitoring (SIM) in the electron ionization (EI) mode can determine concentrations of alachlor, acetochlor, and metolachlor and other major corn herbicides in raw and finished surface water and groundwater samples. This GC/MS method eliminates interferences and provides similar sensitivity and superior specificity compared with conventional methods such as GC/ECD or GC/NPD, eliminating the need for a confirmatory method by collection of data on numerous ions simultaneously. If there are interferences with the quantitation ion, a confirmation ion is substituted for quantitation purposes. Deuterated analogs of each analyte may be used as internal standards, which compensate for matrix effects and allow for the correction of losses that occur during the analytical procedure. A known amount of the deuterium-labeled compound, which is an ideal internal standard because its chemical and physical properties are essentially identical with those of the unlabeled compound, is carried through the analytical procedure. SPE is required to concentrate the water samples before analysis to determine concentrations reliably at or below 0.05 $\mu g\,L^{-1}$ (ppb) and to recover/extract the various analytes from the water samples into a suitable solvent for GC analysis.

DropBooks

3.2.2 Metabolites

The method for chloroacetanilide soil metabolites in water determines concentrations of ethanesulfonic acid (ESA) and oxanilic acid (OXA) metabolites of alachlor, acetochlor, and metolachlor in surface water and groundwater samples by direct aqueous injection LC/MS/MS. After injection, compounds are separated by reversed-phase HPLC and introduced into the mass spectrometer with a TurboIonSpray atmospheric pressure ionization (API) interface. Using direct aqueous injection without prior SPE and/or concentration minimizes losses and greatly simplifies the analytical procedure. Standard addition experiments can be used to check for matrix effects. With multiple-reaction monitoring in the negative electrospray ionization mode, LC/MS/MS provides superior specificity and sensitivity compared with conventional liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/ultraviolet detection (LC/UV), and the need for a confirmatory method is eliminated. In summary,

this technique allows for the separation and quantitation of a complex array of soil metabolites in water samples.

3.3 Description of methodology

3.3.1 Parent compounds

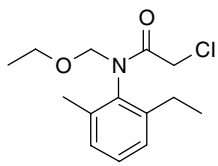
The multi-residue method for alachlor, acetochlor, and metolachlor determines the concentrations of these analytes in groundwater and surface water. This method involves the addition of a deuterated internal standard to the water sample, concentration of the analytes on an SPE column, elution of analytes, concentration of the eluate to a set volume, and analysis by GC/MS. The use of internal standards compensates for recovery losses, but separate experiments were conducted to ensure that recoveries were within 70–120%. The method was validated over the range of 0.05–20 $\mu g\,L^{-1}$ for all components and is presented in Section 6.

3.3.2 Metabolites

The direct aqueous injection and LC/MS/MS multi-residue method determines alachlor, acetochlor, metolachlor and their soil metabolites in water samples. The accuracy of the analytical method is estimated based on the recovery of known concentrations of each metabolite fortified into control water samples that are then carried through the analytical procedure. The method was validated over the range 0.25–20 $\mu g\,kg^{-1}$ for all components and is presented in Section 7.

4 Analytical method for the determination of acetochlor and its metabolites in plants and animals

Identification/properties of acetochlor

Chemical name (IUPAC)	2-Chloro-*N*-ethoxymethyl-6′-ethylacet-*o*-toluidide
Structural formula	
Empirical formula	$C_{14}H_{20}ClNO_2$
Molecular mass	269.8
Melting point	10.6 °C
Boiling point	172 °C
Vapor pressure	0.0046 Pa at 25 °C
Solubility	Water 233 mg L^{-1} (25 °C) Miscible in organic solvents

4.1 Outline of method

Acetochlor and its metabolites are extracted from plant and animal materials with aqueous acetonitrile. After filtration and evaporation of the solvent, the extracted residue is hydrolyzed with base, and the hydrolysis products, EMA and HEMA (Figure 1), are steam distilled into dilute acid. The distillate is adjusted to a basic pH, and EMA and HEMA are extracted with dichloromethane. EMA and HEMA are partitioned into aqueous–methanolic HCl solution. Following separation from dichloromethane, additional methanol is added, and HEMA is converted to methylated HEMA (MEMA) over 12 h. The pH of the sample solution is adjusted to the range of the HPLC mobile phase, and EMA and MEMA are separated by reversed phase HPLC and quantitated using electrochemical detection.

4.2 Apparatus

Meat grinder
Polytron homogenizer
Explosion-proof blender and jars
Analytical and top-loading balances
Polypropylene bottles and seal caps, 25-mL
Superspeed automatic refrigerated centrifuge
pH meter and electrode
Shaker and shaker head
Separatory funnel holder
Rotary evaporator:
- Vacuum trap (one for every two rotary evaporators)
- Adapters
- Evaporator motor
- Hot-plate
- Stainless-steel bowls
- Vacuum pump
- Vacuum tubing
- Ball valve
- Metering valve

Hydrolysis unit (see Figure 2):
- Separatory funnel: Ace Glass No. 7229-08
- Claissen adapter: Ace Glass No. 5055-10
- Straight adapter: Ace Glass No. 5035-10
- Connecting adapter: Ace Glass No. 5125-10
- Liebig condenser: Ace Glass No. 5998-12
- Hose adapter: Ace Glass No. 5217-10
- Heat-shrink Teflon: Cole-Parmer No. L-6851-30
- Trubore tube: Ace Glass No. 8700-41
- Teflon sleeve: Ace Glass No. 7643-08
- Spring tension clip: Ace Glass No. 7600-25

Tygon tubing

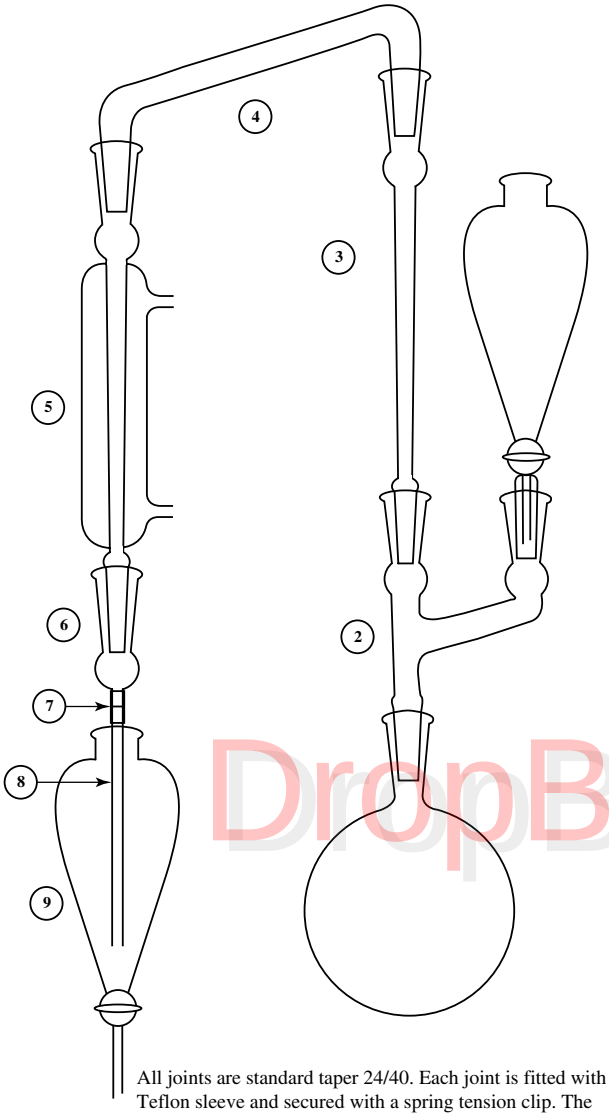
DropBooks

Hemispherical heating mantle
Laboratory jack
Magnetic stirring motor
Variable transformer
Teflon egg-shaped stir bar
Buchner funnel, 85-mm
Glass-fiber filter, 7-cm
Vacuum filtration adapter
Round-bottom flask, 500-mL
Separatory funnels, 125- and 250-mL
Graduated cylinder, 25-mL
Graduated centrifuge tubes, 5- and 10-mL
Pasteur pipets, 5.75- and 9-in lengths
Serological pipets, from 0.1- through 10-mL
Nylon 66 filter membrane, 0.2-μm
Automatic sampler equipped with a 50-μL injection loop
Zorbax C-8 analytical column (15 cm × 4.6-mm i.d.)
In-line high-pressure pre-filter assemblies with ESA carbon filter element
Model 5100A Coulochem Detector with Model 5010 analytical cell and Model 5020guard cell: ESA, Inc.
Pulse damper
HPLC pump
Volumetric flask, 100-mL
HPLC autosampler with Teflon septa

4.3 Reagents

The following reagents have been tested for use in this method. Specific brands are listed to aid in finding suppliers.

Acetonitrile: Fisher No. A-998
Dichloromethane: Fisher No. D-142
Mobile phase: The HPLC mobile phase is made up as follows. Prepare 2 L of acetate buffer by dissolving 13.6 g of sodium acetate and 6 mL of glacial acetic acid in 2 L of deionized water. Adjust the solution to pH 4.8 with concentrated sodium hydroxide solution (or glacial acetic acid) if necessary. Mix 2 L of buffer with 1.6–2 L (the amount depends on the particular commodity) of methanol. Filter the solution through a 0.22-μm Nylon 66 filter membrane before using the mobile phase
Absolute ethanol: Aaper Alcohol and Chemical Co. (200 proof)
Methanol: Fisher No. A-452
Sulfuric acid, 2.5 N: Fisher No. SO-A-208
Sodium hydroxide solution, 50% (w/w): Fisher No. SO-S-254
Igepal CO-660: GAF Corp. [by request; Tel. (+)1 800 622 4423]
Deionized water from a Milli-Q water purification system (Millipore Co.)
Acetonitrile–water (4 : 1, v/v): dilute 200 mL of water to 1 L with acetonitrile
Dow Corning Antifoam B emulsion: Fisher No. CS-283-4



1 125-mL Separatory Funnel
2 Claissen Adapter
3 Straight Adapter
4 Connecting Adapter
5 Leibig Condenser
6 Hose Adapter
7 Heat Shrink Teflon
8 Trubore Tube
9 Collection Funnel
125-mL Separatory Funnel

All glassware obtained from Ace Glass, Inc.

All joints are standard taper 24/40. Each joint is fitted with a Teflon sleeve and secured with a spring tension clip. The delivery tube (8) is joined to the hose barb (6) with a 2 inch length of Teflon heat shrink tubing.

DropBooks

Figure 2 Hydrolysis unit

Concentrated hydrochloric acid: Fisher No. A466-500
Concentrated acetic acid: Fisher No. A465-250
Sodium acetate: Fluka No. 71183
4 N Hydrochloric acid: dilute 330 mL of HCl to 1 L with deionized water
4 N Hydrochloric acid–methanol (10 : 3, v/v): prepare 1 L of solution by combining 770 mL of 4 N HCl with 230 mL of methanol
Igepal CO-660, 10% (v/v) in water: dilute 100 mL of Igepal to 1 L with water
Hexane: Fisher No. H-302

4.4 Analytical standards

Analytical standards are prepared for two purposes: for fortifying control matrices to determine the analytical accuracy and for calibrating the response of the analyte in the electrochemical detector.

6-Ethyl-*o*-toluidine
2-Ethyl-6-methylaniline (EMA)

The following standards were synthesized by Monsanto Company and are available through the Environmental Protection Agency National Pesticide Standard Repository (Fort Meade, MD, USA):

2-(1-Hydroxyethyl)-6-methylaniline, >97% pure (HEMA)
Acetic acid, [(ethoxymethyl)(2-ethyl-6-methylphenyl)amino]oxo-, sodium salt, should be >95% pure (EMA-producing oxanilic acid metabolite, referred to from this point as Metabolite **I**)

Ethanesulfonic acid, 2-[(ethoxymethyl)[2-(1-hydroxyethyl)-6-methylphenyl] amino]-2-oxo-, sodium salt, hydrate (1 : 1), should be >95% pure (HEMA-producing sulfonic acid metabolite, referred to from this point as Metabolite **II**)

Ethanesulfonic acid, 2-[(2-ethyl-6-methylphenyl)(ethoxymethyl)amino]-2-oxo-, sodium salt, should be >95% pure (EMA-producing sulfonic acid metabolite, referred to from this point as Metabolite **III**)

Acetic acid, [(ethoxymethyl)[2-(1-hydroxyethyl)-6-methylphenyl]amino]oxo-, sodium salt, should be >95% pure (HEMA-producing oxanilic acid metabolite, referred to from this point as Metabolite **IV**)

4.4.1 Fortification solutions for animal commodities

Weigh the appropriate amount of Metabolites **I** and **II** separately into 100-mL volumetric flasks. If the purity of the metabolite standard is ≤95%, but at least ≥90%, adjust the target weight of the standard to compensate for the purity. If the purity is <90%, this standard should not be used. Dilute the standard to volume with absolute ethanol, and mix the solution well to ensure complete dissolution. These solutions contain 1000 μg mL^{-1} in acetochlor equivalents of each metabolite. From these solutions, prepare mixed metabolites solutions at final concentrations of 0.5, 1.0, 2.0, 5.0, and 10.0 μg mL^{-1}. Store all standards refrigerated (0–6 °C) in amber-glass bottles.

4.4.2 Fortification solutions for plant commodities

Weigh the appropriate amount of Metabolites **III** and **IV** separately into 100-mL volumetric flasks. If the purity of the metabolite standard is ≤95%, but at least ≥90%, adjust the target weight of the standard to compensate for the purity. If the purity is <90%, this standard should not be used. Dilute the standard to volume with absolute ethanol, and mix the solution well to ensure complete dissolution. These solutions contain 1000 μg mL^{-1} in acetochlor equivalents of each metabolite. From these solutions, prepare mixed metabolite solutions at final concentrations of 0.5, 1.0, 2.0, 5.00 and 10.0 μg mL^{-1}. Store all standards refrigerated (0–6 °C) in amber-glass bottles.

4.4.3 Detector calibration standards

Weigh 0.1000 g of analytical standard-grade EMA into a 100-mL volumetric flask, dilute the standard to volume with analytical-grade methanol, and mix the solution well. This solution contains 1000 μg mL^{-1} of EMA.

Weigh 0.1000 g of analytical standard-grade HEMA into another 100-mL volumetric flask, dilute the standard to volume with analytical-grade methanol, and mix the solution well. This solution contains 1000 μg mL^{-1} of HEMA.

Pipet 10.0 mL of each of the above solutions into a 100-mL volumetric flask, dilute the mixture of solutions to volume with methanol, and mix the resulting solution well. This solution contains 100 μg mL^{-1} of each of the standards.

In addition, prepare 10.0, 1.0, and 0.10 μg mL^{-1} standards.

All standards are stored refrigerated (0–6 °C) in amber-glass bottles. The EMA and HEMA calibration standards have been shown to be stable in methanol for 6 months when stored refrigerated.

Seven standards are prepared from the above HEMA/EMA standard solutions typically with every sample set. The preparation of the detector calibration standards, in this way, accounts for the completeness of the HEMA conversion to MEMA.

In separate 25-mL graduated cylinders, add 16 mL of the 4 N HCl–methanol solvent mixture (10 : 3) and the quantities of the EMA/HEMA standard solution in methanol given in Table 2.

Add methanol to each cylinder to bring the volume up to 20 mL. Carry out the methylation of the standards in the same manner and at the same time as the samples. Ultimately, the standards are diluted to 25 mL. For the resulting EMA and MEMA concentrations, see Table 2.

Note: molecular weights used in conversions from acetochlor to the anilines are as follows: acetochlor, 269.77; EMA, 135.21; HEMA, 151.21; and MEMA, 165.24.

DropBooks

4.5 Analytical procedure

4.5.1 Sample preparation

Milk is homogenized in the original container by shaking the container vigorously. Muscle, liver, kidney, and fat are ground partially frozen in a meat grinder. Plant commodities are ground while frozen with dry-ice in a blender or chopper. The dry-ice will sublime in a cold room overnight.

Table 2 Final calibration standards

Standard No.	Volume of standard solution (mL)	Final EMA concentration (μg mL^{-1})	Final MEMA concentration (μg mL^{-1})
1	None	0.00000	0.00000
2	0.5 (0.1 μg mL^{-1})	0.00200	0.00219
3	1.0 (0.1 μg mL^{-1})	0.00400	0.00437
4	2.0 (0.1 μg mL^{-1})	0.00800	0.00874
5	0.5 (1.0 μg mL^{-1})	0.02000	0.02186
6	1.0 (1.0 μg mL^{-1})	0.04000	0.04371
7	2.0 (1.0 μg mL^{-1})	0.08000	0.08742

4.5.2 Sample extraction

(1) Plant material. Weigh 25 g of the chopped and frozen sample into a blender jar. To check recoveries, spike the fortification samples with the appropriate volume of metabolite standard at this point. Add 200 mL of acetonitrile–water (4 : 1, v/v) to the jar, and blend the sample at medium speed for 5 min. Filter the extract through a Buchner funnel fitted with a glass-fiber filter pad into a 500-mL round-bottom flask containing 10 drops of Antifoam B and 3 mL of 10% aqueous Igepal CO-660 (nonionic surfactant). The flask is connected to the Buchner funnel by means of an adapter suitable for applying vacuum to the system.

Rinse the blender jar and filter cake twice with 25–50-mL portions of 20% water–acetonitrile, collecting each rinse in the round-bottom flask.

(2) Milk. Shake the sample vigorously, and immediately weigh 25 g of milk into a 250-mL centrifuge bottle. Make fortifications to the samples at this point. Add 200 mL of acetonitrile, balance duplicate bottles for later centrifugation of samples, cap and shake the samples for 20 min on a mechanical shaker. Centrifuge the samples for 15 min at 11 000 rpm. Decant the aqueous acetonitrile supernatant from each sample into a 500-mL round-bottom flask through a funnel, leaving any viscous fluid that may be present in the bottom of the bottle. Add 10 drops of Dow Corning Antifoam B emulsion and 3 mL of 10% aqueous Igepal CO-660 (nonionic surfactant) to the flask.

(3) Fat. Weigh 25 g of fat into a 250-mL polypropylene centrifuge bottle. Make fortifications to the samples at this point. Add 100 mL of hexane to the sample, and homogenize the sample with a Polytron at high speed for 1 min. Add an additional 100 mL of acetonitrile–water (4 : 1, v/v), and continue homogenization of the sample with the Polytron until the sample is thoroughly mixed. Balance duplicate centrifuge bottles, and cap and shake the bottles on a mechanical shaker for 30 min. Centrifuge the samples at 11 000 rpm for 15 min. Decant the supernatant through a powder funnel plugged with glass-wool into a 250-mL separatory funnel. Allow 15 min for phase separation. Save the centrifuge bottle for extraction.

Drain the aqueous acetonitrile (lower) phase into a 500-mL round-bottom flask, and save the separatory funnel for extraction. Extract the hexane–fat mixture by transferring the mixture back to the polypropylene centrifuge bottle and adding 100 mL of acetonitrile–water (4 : 1, v/v) solution. Balance the duplicate centrifuge bottles, and cap and shake the bottles for 10 min on the shaker. Centrifuge the second extract at 11 000 rpm for 15 min. Decant this second extract into the 250-mL separatory funnel as before. After phase separation, combine the aqueous extracts in the 500-mL round-bottom flask, and discard the top hexane–fat layer. Add 10 drops of Dow Corning Antifoam B emulsion and 3 mL of 10% aqueous Igepal CO-660 (nonionic surfactant) to the flask.

(4) Kidney, liver, and muscle. Weigh 25 g of sample into a 250-mL polypropylene centrifuge bottle. Make fortifications to the samples at this point. Add 100 mL of acetonitrile–water (4 : 1, v/v) and 10 drops of Dow Corning Antifoam B emulsion to the sample bottles, and homogenize the samples with a Polytron at high speed for 1 min. Rinse the Polytron probe with an additional 80 mL of acetonitrile–water

(4 : 1, v/v), collecting the rinse in the original sample bottle. Balance duplicate centrifuge bottles with acetonitrile–water (4 : 1, v/v), and cap and shake the bottles on a mechanical shaker for 20 min. Centrifuge the samples at 11 000 rpm for 20 min. Decant the supernatant aqueous acetonitrile through a powder funnel into a 500-mL round-bottom flask. Add 10 drops of Dow Corning Antifoam B emulsion and 3 mL of 10% aqueous Igepal CO-660 (nonionic surfactant) to the flask.

4.5.3 Concentration by rotary evaporation

Attach the 500-mL round-bottom flask containing the filtered extract to a rotary evaporator connected to a vacuum pump with the flask immersed in a room temperature water-bath. Under reduced pressure, the sample extract will have a tendency to foam or 'bump' owing to degassing of the solvent. During concentration under vacuum, do not allow the extract to foam or boil out of the flask. To do this, apply partial vacuum to the sample until the extraction solvent has been degassed. Then, over a 5–10-min period, increase the vacuum on the sample using the vacuum-metering valve, slowly closing this valve to gradually allow the sample to be concentrated under full capacity of the vacuum pump. Gradually, over a 20–30-min period, increase the water-bath temperature of the rotary evaporator to a maximum of 40 °C after full vacuum has been applied to the system. Continue the evaporation of the solvent until the extract has been reduced to 3–5 mL. The sample will usually have the consistency of an oily viscous liquid.

4.5.4 Residue hydrolysis

DropBooks

A hydrolysis unit is constructed using the commercially available glassware shown in Figure 2. The collection funnel should be calibrated and marked for volumes of 35 and 85 mL. All 20/40-glass joints of the hydrolysis unit should be assembled with Teflon sleeves and secured with spring clamps to prevent loss of the volatile EMA and HEMA analytes.

Place a Teflon, egg-shaped, magnetic stirring bar in the 500-mL round-bottom flask containing the sample extract, and attach the flask to the hydrolysis unit. Position a variable transformer-controlled heating mantle under the flask. The heating mantle is supported between the flask and the magnetic stirring motor by an adjustable laboratory jack. Add 10 mL of 2.5 N sulfuric acid to a 125-mL separatory funnel. Position this funnel (item 9, Figure 2) such that the delivery tip of the hydrolysis unit (item 8, Figure 2) is about 1 in beneath the surface of the acid. Turn on the cooling water to the distillation condenser. Start the magnetic stirring motor so that the stir bar is gently spinning continuously. Do not spin the stir bar too fast, as excessive sample foaming may result. By opening the stopcock completely, quickly add 50 mL of 50% sodium hydroxide to the 500-mL round-bottom flask via the side-arm addition funnel of the hydrolysis unit (item 1, Figure 2). Close the addition funnel stopcock immediately after the base has been added. Add 50 mL of deionized water to the addition funnel with the stopcock in a closed position. Turn on the variable transformer controlling the heating mantle. Adjust the power output of the transformer to allow the sample to come to a boil, and distill the solvent. Do not overheat the sample mixture. The transformer setting is approximately 90–100. After 25 mL of the distillate have been collected (25 mL of distillate plus the original 10 mL of 2.5 N sulfuric acid = 35 mL

total volume), slowly add the 50 mL of deionized water through the side-arm addition funnel while continuing the distillation. To minimize cooling of the flask and back-siphoning of the distillate, the water must be added drop-wise over approximately a 10–15-min period. Additionally, the collection funnel must be lowered so that the delivery tip of the hydrolysis unit is approximately 0.25 in beneath the surface of the liquid. This will minimize the amount of distillate that can be drawn up into the unit during this step but will still allow the analyte to be trapped during the continued distillation. Close the addition funnel stopcock immediately after the deionized water has been added. Continue the distillation until the total volume in the collection funnel is 85 mL (75 mL of distillate plus the original 10 mL of 2.5 N sulfuric acid). Lower the receiving funnel until the delivery tip of the hydrolysis unit just touches the surface of the liquid (this will prevent the distillate from back-siphoning when the heat is removed). Turn off the variable transformer, and remove the heating mantle from the round-bottom flask. Slowly, over a 10–15-min period, add approximately 100 mL of deionized water to the flask through the side-arm addition funnel to dilute the highly concentrated caustic. The water must be added slowly to avoid a violent reaction with the hot concentrated caustic. Owing to the corrosive effects of caustic on glass, the 500-mL round-bottom flasks should be discarded after a maximum of three uses.

This is a convenient overnight stopping point if necessary. Stopper the separatory funnel containing the acidic distillate, and store the distillate at room temperature until analysis can be resumed the next day.

4.5.5 Analyte extraction and conversion of HEMA to MEMA

To the acidic distillate in the 125-mL separatory funnel, add 5 mL of 50% sodium hydroxide and 15 mL of dichloromethane. Cap the separatory funnel tightly, and allow its contents to cool for 30 min. Heat created by the addition of caustic to the acidic distillate will cause some of the dichloromethane to volatilize, creating pressure in the funnel; therefore, the cap must be secured tightly to the funnel. Escaping solvent will result in loss of analytes. Shake the funnel for 5 min on a mechanical shaker. Allow 15 min for phase separation after shaking the funnel. Drain the lower dichloromethane layer into a second 125-mL separatory funnel. Extract the aqueous layer a second time with 15 mL of dichloromethane. Following shaking of the funnel and phase separation, combine both dichloromethane layers in the same 125-mL separatory funnel.

Extract the combined dichloromethane portions with 2×8 mL of a mixture of 4 N HCl–methanol (10 : 3, v/v). Cap the separatory funnel, and shake the funnel manually, venting the pressure 2–3 times before shaking the funnel for 5 min on a mechanical shaker. Allow 15 min for phase separation after shaking. Discard the lower dichloromethane phase. Drain the aqueous phase into a 25-mL graduated cylinder. Add 4 mL of methanol, cap the graduated cylinder, and shake the cylinder gently by inverting it several times to ensure complete mixing. The samples and freshly prepared standards are reacted at room temperature overnight (12 h minimum). This step converts HEMA into MEMA. After the reaction, cool the graduated cylinders for 15 min in an ice-bath. Add 2.3 mL of 50% NaOH to the cylinder, replace the stopper, and allow the contents of the cylinder to cool for an additional 15 min. Mix the sample by inverting the graduated cylinder several times. Transfer the sample into a 50-mL beaker, and gently mix the sample in the beaker using a stir plate and a small stirring bar. Remove the samples and/or standards from the ice-bath one at a time for pH

adjustment. Warm samples will result in loss of analytes. Adjust the pH of the acidic sample by dropwise addition of 50% NaOH. Monitor the pH using a meter calibrated with pH 7 and pH 4 buffers. Bring the final pH of the sample to 6.0 using dilute (1.0, 0.1 or 0.01 N) NaOH or HCl. Transfer the sample back in to the 25-mL graduated cylinder. Rinse the beaker with small volumes of methanol–water (1:1, v/v) solvent mixture, and transfer the rinsate into the cylinder until the final adjusted volume is 25 mL (at room temperature). Mix the sample thoroughly by repeatedly inverting the graduated cylinder. The sample is now ready for separation and quantitation by HPLC/OECD. Samples that cannot be analyzed within an 8-h period after pH adjustment must be refrigerated, as some breakdown of the MEMA derivative may occur.

4.6 *Instrumentation*

The analytes, EMA and MEMA, are separated and quantitated by reversed phase HPLC/OECD. Details of the operating conditions are as follows:

HPLC/OECD operating conditions

Flow rate	1.0 mL min^{-1}
Mobile phase	Acetate buffer (pH 4.8)–MeOH (1 : 1, v/v), proportions may change depending on the matrix
Column	DuPont Zorbax C-8 (150 mm × 4.6-mm i.d.)
Temperature	Ambient
*Detection**	Operate in the screen mode Guard cell potential = +0.90 V Detector 1 = +0.30 V Detector 2 = +0.75 V
Gain	200
Response time	0.4 s
Sample size	50 μL

*Optimum detector settings may vary from instrument to instrument; therefore, minor adjustments may be made to optimize performance.

DropBooks

The electrochemical detector must be zeroed after each analysis just before the next sample injection. This procedure is necessary owing to the drifting baseline associated with the electrochemical detector. The detector is equipped with this baseline zero capability, and the adjustment can be activated through an external event output signal sent from an autosampler.

Instrument calibration is done during the analysis of samples by interspersing standards among the samples. Following completion of the samples and standards, a linear calibration curve is estimated from the response of the standards using standard linear regression techniques. The calibration constants obtained from each run are used only for the samples quantitated in that run. Drastic changes or lack of linearity may indicate a problem with the detector.

4.7 *Calculation of residues*

The amounts of EMA and HEMA (derivatized as MEMA) are determined based upon external standard calibration. A nonweighted linear least-squares estimate of

the calibration curve is used to calculate the amount of EMA and MEMA in the unknowns. The response of any given sample should not exceed the response of the most concentrated standard.

A new nonweighted linear calibration curve is to be generated with every set of samples analyzed. The calibration standards are interspersed among the analytical samples, preferably with a standard between every two analytical samples, and injected into the HPLC/OECD system. The calibration curve is generated by plotting peak height of the detector response against the concentration for each calibration standard of EMA and methylated HEMA.

The amount of EMA and MEMA determined is converted to the equivalent amount of acetochlor for reporting purposes. This is readily accomplished using the following equations:

$$\frac{\mu g\,\text{EMA found}}{\text{Sample mass (g)}} \times 1.995 = \text{mg kg}^{-1}\text{acetochlor}$$

$$\frac{\mu g\,\text{MEMA found}}{\text{Sample mass (g)}} \times 1.633 = \text{mg kg}^{-1}\text{acetochlor}$$

The conversion factor, 1.995, corrects for the difference in molecular weight between acetochlor (269.77) and EMA (135.21). The conversion factor, 1.633, corrects for the difference in molecular weight between acetochlor (269.77) and MEMA (165.24).

4.8 Evaluation

4.8.1 Modifications or potential problems

EMA is volatile. During the hydrolysis/distillation procedure, the delivery tip of the hydrolysis unit must remain below the surface of the liquid in the receiving funnel at all times. Procedures that involve generation of heat must be performed carefully to minimize losses.

The HPLC elution pattern is affected to some extent by the pH of the mobile phase. Moderate pH adjustment to optimize the resolution between EMA and MEMA may be performed. Retention time can be affected greatly by the history of the HPLC column and also the buffer/methanol ratio. The mobile phase ratio should be adjusted to provide adequate separation and retention. Control and fortified samples should be run in the same analytical set with treated samples.

4.8.2 Recoveries, limits of detection and quantitation

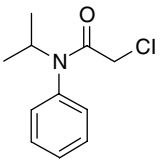
The method was validated in numerous matrices, both animal and crop, at levels ranging from 0.01 to 0.50 mg kg^{-1} in acetochlor equivalents for each of the two metabolite analytes. Analytical recoveries were >70%. No apparent trends were observed for either the level of fortification or the matrix analyzed.

The limit of detection (LOD) and limit of quantitation (LOQ) were statistically determined in alfalfa and clover raw agricultural commodities (rotational crops). The method LOD and LOQ for the EMA-producing metabolite were 0.004 and 0.012 mg kg^{-1}, respectively, and the LOD and LOQ for the HEMA-producing metabolite were 0.004 and 0.014 mg kg^{-1}, respectively, for the alfalfa and clover

RACs. The LOD and LOQ for each study should be determined. Until each study's LOD and LOQ have been determined, use the lower limit of method validation (LLMV) of 0.01 mg kg^{-1} for each metabolite class as the reference LOQ. LLMV is defined as the lowest level of fortification where we have demonstrated acceptable recovery and precision of EMA- and HEMA-producing metabolites.

5 Analytical method for the determination of propachlor and its metabolites in plants and animals

Identification/properties of propachlor

Chemical name (IUPAC)	2-Chloro-*N*-isopropylacetanilide
Structural formula	
Empirical formula	$C_{11}H_{14}ClNO$
Molecular mass	211.69
Melting point	77–78 °C
Boiling point	110 °C
Vapor pressure	0.01 Pa at 25 °C
Solubility	Water 613 mg L^{-1} (25 °C) Ethanol 290 g L^{-1} (20 °C) Carbon tetrachloride 148 g L^{-1} (20 °C) Xylene 193 g L^{-1} (20 °C)

DropBooks

5.1 Outline of method

Propachlor is extracted from plant and animal material with aqueous acetonitrile. After filtration and evaporation of the solvent, the extracted residue is hydrolyzed with base, and the hydrolysis product, NIPA, is steam distilled into dilute acid. The acid distillate is partitioned with dichloromethane. The aqueous layer is adjusted to a basic pH, and NIPA is extracted with isooctane. The extracted residues are cleaned up using a silica SPE column, and NIPA is eluted with isooctane–ethyl acetate (9 : 1, v/v) solvent mixture. Quantitation is by GC/NPD.

5.2 Apparatus

Meat grinder
Hobart chopper
Polytron homogenizer
Explosion-proof blender and jars

Top-loading and analytical balances
Polypropylene bottles and seal caps, 250-mL
Superspeed automatic refrigerated centrifuge
Shaker and shaker head
Hemispherical heating mantle
Laboratory jack
Magnetic stirring motor
Variable transformer
Teflon egg-shaped stir bar
Tygon tubing
Rotary evaporator (see corresponding section in previous method)
Hydrolysis unit (see Figure 2, corresponding section in previous method)
Separatory funnel holder
Buchner funnel, 85-mm
Glass-fiber filter, 7-cm
Vacuum filtration adaptor
Round-bottom flask, 500-mL
Separatory funnel, 125-mL
Volumetric flask, 100-mL
Graduated centrifuge tubes, 5- and 13-mL
Pasteur pipets, 5.75- and 9-in lengths
Serological pipets, from 0.1- through 5-mL
Solid-phase extraction columns, 6-mL silica
Vacuum manifold
Gas chromatograph equipped with a thermionic specific detector (TSD)
DB-5 Megabore capillary column, 30 m × 0.53-mm i.d.

5.3 Reagents

The following reagents have been tested for use in this method. Specific brands are listed as an aid to find suppliers.

Acetonitrile: Fisher No. A-996-4
Dichloromethane: Fisher No. D-142
Absolute ethanol: Aaper Alcohol and Chemical Co. (200 proof)
Ethyl acetate, Fisher No. E-195
Isooctane, Fisher No. 0301-4
Sulfuric acid, 2.5 N: Fisher No. SA-208
Sodium hydroxide solution, 50% (w/w): Fisher No. SS-254
Anhydrous sodium sulfate, Fisher No. S421-500
Deionized water from a Milli-Q water purification system (Millipore Co.)
Igepal CO-660: GAF Corp. (by request; Tel. (+)1 800 622 4423)
Acetonitrile–water (4 : 1, v/v): dilute 200 mL of deionized water to 1 L with acetonitrile
Isooctane–ethyl acetate (9 : 1, v/v): dilute 100 mL of ethyl acetate to 1 L with isooctane
Dow Corning Antifoam B emulsion: Fisher No. CS-283-4

5.4 Analytical standards

Analytical standards are prepared for two purposes: for fortifying control matrices to determine the analytical accuracy and for calibrating the response of the analyte in the nitrogen-sensitive detector.

The following analytical standards were synthesized by Monsanto Company and may be available through the Environmental Protection Agency, National Pesticide Standard Repository (Fort Meade, MD, USA): [1-(methylethyl)phenylamino] oxoacetic acid, sodium salt, >95% pure (NIPA-producing metabolite) and *N*-isopropylaniline (NIPA), >99% pure.

5.4.1 Fortification solutions

Weigh 0.1 g in propachlor equivalents of the representative NIPA-producing metabolite into a 100-mL volumetric flask. Dilute the standard to volume with absolute ethanol, and mix the solution well to ensure complete dissolution. This solution contains 1000 μg mL^{-1} propachlor equivalents of NIPA-producing metabolite. Dilute this solution as appropriate to prepare fortification standards at the following concentrations: 0.10, 0.50, 1.0, 2.0, 5.0, 25, and 50-μg mL^{-1}. Store all standards refrigerated (0–6 °C) in amber-glass bottles.

5.4.2 Detector calibration standards

Weigh 0.1000 g of analytical standard NIPA into a 100-mL volumetric flask, dilute the standard to volume with isooctane–ethyl acetate (9 : 1, v/v), and mix the solution well. This solution contains 1000 μg mL^{-1} of the analyte. Dilute the solution as appropriate to prepare calibration standards at the following concentrations: 0.01, 0.025, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00 μg mL^{-1}. Store all standards refrigerated (0–6°C) in amber-glass bottles.

DropBooks

5.5 Analytical procedure

5.5.1 Sample preparation

Milk is homogenized in the original container by shaking the container vigorously. Muscle, liver, kidney and fat are ground partially frozen in a meat grinder. Plant commodities are ground while frozen with dry-ice in a blender or chopper. The dry-ice will sublime in a cold room overnight.

5.5.2 Sample extraction

(1) Plant material. Weigh 25 g of the chopped and frozen sample into a blender jar. To confirm recoveries, prepare fortification samples by spiking the matrix with the appropriate volume of metabolite standard. Add 200 mL of acetonitrile–water (4 : 1, v/v) solution to the jar, and blend the mixture at medium speed for 5 min. Filter the extract through a Buchner funnel fitted with a glass-fiber filter pad into a 500-mL round-bottom flask containing 10 drops of Antifoam B and 3 mL of 10% aqueous Igepal CO-660 (nonionic surfactant). The flask is connected to the Buchner funnel by

means of an adapter suitable for applying vacuum to the system. Rinse the blender jar and filter cake twice with 25–50-mL portions of acetonitrile–water (4 : 1, v/v), collecting each rinse in the round-bottom flask.

(2) Milk. Shake the sample vigorously, and immediately weigh 25 g of milk into a 500-mL round-bottom flask. Add 10 drops of Dow Corning Antifoam B emulsion and 3 mL of 10% aqueous Igepal CO-660 (nonionic surfactant) to the flask. Fortify the sample in the flask.

(3) Eggs. Blend several eggs briefly in a blender, and weigh 25 g into a 250-mL polypropylene centrifuge bottle. Fortify the sample with standard at this point. Add 200 mL of acetonitrile–water (4 : 1, v/v) solution, cap the bottle tightly, and shake the mixture on a mechanical shaker for 5 min. Centrifuge samples at 10 000 rpm for 15 min. Decant the supernatant aqueous acetonitrile through a Buchner funnel into a 500-mL round-bottom flask. Add 10 drops of Dow Corning Antifoam B emulsion and 3 mL of 10% aqueous Igepal CO-660 (nonionic surfactant) to the flask.

(4) Animal tissues. Weigh 25 g of appropriate animal tissue into a 250-mL polypropylene centrifuge bottle. Fortify the samples with standards at this point. Add 200 mL of acetonitrile–water (4 : 1, v/v) to the bottle, and homogenize the mixture with a Polytron at medium speed for 5 min. Centrifuge the samples at 10 000 rpm for 15 min. Decant the supernatant aqueous acetonitrile through a Buchner funnel into a 500-mL round-bottom flask. Add 10 drops of Dow Corning Antifoam B emulsion and 3 mL of 10% aqueous Igepal CO-660 (nonionic surfactant) to the flask.

5.5.3 Concentration by rotary evaporation

Attach the 500-mL round-bottom flask containing the filtered extract to a rotary evaporator connected to a vacuum pump with the flask immersed in a room-temperature water-bath. Under reduced pressure, the sample extract will have a tendency to foam or 'bump' owing to degassing of the solvent. During concentration under vacuum, do not allow the extract to foam or boil out of the flask. To do this, apply partial vacuum to the sample until the extraction solvent has been degassed. Then, over a 5–10-min period, increase the vacuum on the sample using the vacuum-metering valve, slowly closing this valve to allow the sample to be concentrated gradually under the full capacity of the vacuum pump. Gradually, over a 20–30-min period, increase the water-bath temperature of the rotary evaporator to a maximum of 40 °C after full vacuum has been applied to the system. Continue the evaporation until the extract has been reduced to 2–3 mL. The sample will usually have the consistency of an oily viscous liquid.

5.5.4 Residue hydrolysis

A hydrolysis unit is constructed using the commercially available glassware shown in Figure 2. The collection funnel should be calibrated and marked for volumes of 35 and 85 mL. All 20/40-glass joints of the hydrolysis unit should be assembled with Teflon sleeves and secured with spring clamps to prevent loss of the volatile NIPA analyte.

Place a Teflon, egg-shaped, magnetic stirring bar in the 500-mL round-bottom flask containing the sample extract, and attach the flask to the hydrolysis unit. Position a

variable transformer-controlled heating mantle under the flask. The heating mantle is supported between the flask and the magnetic stirring motor by an adjustable laboratory jack. Add 10 mL of 2.5 N sulfuric acid to a 125-mL separatory funnel. Position this funnel (item 9, Figure 2) such that the delivery tip of the hydrolysis unit (item 8, Figure 2) is about 1 in beneath the surface of the acid. Turn on the cooling water to the distillation condenser. Start the magnetic stirring motor so that the stir bar is gently spinning continuously. Do not spin the stir bar too fast, as excessive sample foaming may result. By opening the stopcock completely, quickly add 50 mL of 50% sodium hydroxide to the 500-mL round-bottom flask via the side-arm addition funnel of the hydrolysis unit (item 1, Figure 2). Close the addition funnel stopcock immediately after the base has been added. Add 50 mL of deionized water to the addition funnel with the stopcock in a closed position. Turn on the variable transformer controlling the heating mantle. Adjust the power output of the transformer to allow the sample to come to a boil, and distill the solvent. Do not overheat the sample mixture. The transformer setting is approximately 90–100. After 25 mL of the distillate have been collected (25 mL of distillate plus the original 10 mL of 2.5 N sulfuric acid = 35 mL total volume), slowly add the 50 mL of deionized water through the side-arm addition funnel while continuing the distillation. To minimize cooling of the flask and back-siphoning of the distillate, the water must be added dropwise over approximately a 10–15-min period. Additionally, the collection funnel must be lowered so that the delivery tip of the hydrolysis unit is approximately 0.25 in beneath the surface of the liquid. This will minimize the amount of distillate that can be drawn up into the unit during this step but will still allow the analyte to be trapped during the continued distillation. Close the addition funnel stopcock immediately after the deionized water has been added. Continue the distillation until the total volume in the collection funnel is 85 mL (75 mL of distillate plus the original 10 mL of 2.5 N sulfuric acid). Lower the receiving funnel until the delivery tip of the hydrolysis unit just touches the surface of the liquid (this will prevent the distillate from back-siphoning when the heat is removed). Turn off the variable transformer, and remove the heating mantle from the round-bottom flask. Slowly, over a 10–15-min period, add approximately 100 mL of deionized water to the flask through the side-arm addition funnel to dilute the highly concentrated caustic. The water must be added slowly to avoid a violent reaction with the hot concentrated caustic. Owing to the corrosive effects of caustic on glass, the 500-mL round-bottom flasks should be discarded after a maximum of three uses.

DropBooks

This is a convenient overnight stopping point if necessary. Stopper the separatory funnel containing the acidic distillate, and store the distillate at room temperature until analysis can be resumed the next day.

5.5.5 Analyte extraction

To the acidic distillate in the 125-mL separatory funnel, add 10 mL of dichloromethane. Cap the separatory funnel tightly, and shake the funnel for 5 min on a mechanical shaker. Allow 15 min for phase separation after shaking. Drain and discard the lower dichloromethane layer. Add 5 mL of 50% sodium hydroxide to the aqueous solution in the separatory funnel. Cap the separatory funnel tightly, and allow the contents to cool for 30 min. Heat created by the addition of the caustic to the acidic distillate will cause some of the NIPA to volatilize in the funnel; therefore, the cap of

the funnel must be secured tightly to prevent loss of the analyte. To the basic distillate in the 125-mL separatory funnel, add 10 mL of isooctane, and shake the funnel for 5 min on a mechanical shaker. Allow 20 min for phase separation after shaking the funnel. Drain and discard the lower aqueous layer from the funnel. Collect the isooctane layer (which contains NIPA) in a 13-mL calibrated tube containing a small amount of anhydrous sodium sulfate to absorb any water present.

This is a convenient stopping point if samples cannot be completed. Store samples overnight in a refrigerator at 2–6 °C.

5.5.6 Solid-phase extraction cleanup

Connect a vacuum manifold to a vacuum source not to exceed 20 in Hg, and place a 1-L waste liquid trap between the manifold and the vacuum source. To use the manifold, remove the cover, and place a Luer hub solvent guide needle on the male Luer fitting of each flow control valve. Remove the collecting vessel rack, and replace the cover. Close all control valves on the manifold cover.

Add 0.5–0.75 in of anhydrous sodium sulfate to the head of a 6-mL silica disposable column to ensure removal of any residual water, and attach a 15-mL reservoir to the top of each column. Place the desired number of silica cleanup columns into the female Luer receptacles on the cover. Turn the vacuum on at the source, and set the vacuum to about 10 inHg. Wash each column with isooctane. If the column goes dry, add an additional 10 mL of isooctane. When the solvent in the column reaches the top of the packing bed, turn the flow control valve fully off.

Add the NIPA-containing isooctane sample to the reservoir, and elute the solution dropwise. Add 10 mL of isooctane to rinse the glass tube, and apply this rinse to the column before the column runs completely dry. Next, allow the column to go to dryness for at least 1 min under vacuum. Release the vacuum, and insert the collecting rack with 5-mL tubes. Elute NIPA dropwise with 5 mL of isooctane–ethyl acetate (9 : 1, v/v) under a vacuum of about 10 inHg. After the elution is complete, apply full vacuum (approximately 20 inHg), and allow the columns to drain completely dry. Release the vacuum slowly.

Adjust the volume collected to 5 mL, if necessary, and mix the contents thoroughly. The sample is now ready for separation and quantitation by GC/NPD.

5.6 Instrumentation

The NIPA analyte is separated and quantitated by GC/NPD. Details of the operating conditions are as follows:

GC/NPD operating conditions

Column	J & W Scientific DB-5 Megabore, 30 m × 0.53-mm i.d.
Column temperature	100 °C held for 1 min, then increased at 3 °C min^{-1} to 150 °C (no hold)
Injector temperature	250 °C
Detector temperature	300 °C
Hydrogen flow rate	4.5 mL min^{-1}
Air flow rate	175 mL min^{-1}

Nitrogen flow rate	5 mL min^{-1}
N_2 make-up gas flow rate	25 mL min^{-1}
Attenuation	8
Range	10^{-12}
Injection volume	5.0 μL

Instrument calibration is performed during the analysis of samples by interspersing standards among the samples. Following completion of the samples and standards, a linear calibration curve is estimated from the response of the standards using standard linear regression techniques. The constants obtained from each run are used only for the samples quantitated in that run. While calibrating on such a frequent basis, variations in the values of the slope and intercept of the calibration curve occur. Drastic changes or lack of linearity may indicate a problem with the detector.

5.7 Calculation of residues

The amount of NIPA is determined based upon external standard calibration. A nonweighted linear least-squares estimate of the calibration curve is used to calculate the amount of NIPA in the unknowns. The response of any given sample must not exceed the response of the most concentrated standard. If this occurs, dilution of the sample will be necessary.

A new nonweighted linear calibration curve is to be generated with every set of samples analyzed. The calibration standards are included in the analytical sample set, as the set is injected into the GC system, preferably with a standard between every two analytical samples.

DropBooks

The amount of the analyte determined is converted to the equivalent amount of propachlor for reporting purposes. This is readily accomplished using the following equation:

$$\frac{\mu g \text{ NIPA found}}{\text{sample mass (g)}} \times 1.57 = \text{mg kg}^{-1} \text{ propachlor}$$

The conversion factor, 1.57, corrects for the difference in the molecular weight between propachlor (211.69) and NIPA (135.21).

5.8 Evaluation

5.8.1 Modifications or potential problems

NIPA is volatile. During the hydrolysis/distillation procedure, the delivery tip of the hydrolysis unit must remain below the surface of the liquid in the receiving funnel at all times. Procedures that involve generation of heat must be performed carefully to minimize losses.

Exercise caution during the addition of the water in the distillation phase to prevent aspiration of the distillate back into the 500-mL round-bottom flask.

As a safety precaution, the 500-mL round-bottom flasks should be used a maximum of three times for hydrolysis, after which they should be discarded.

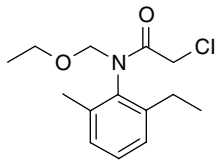
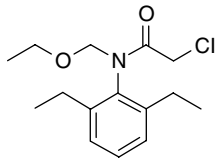
5.8.2 Recoveries, limits of detection and quantitation

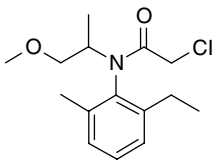
The method was validated in numerous matrices, both animal and crop, at levels ranging from 0.01 to 10 mg kg^{-1} in propachlor equivalents. Analytical recoveries were >70%. No apparent trends were observed for either the level of fortification or the matrix analyzed.

The LOD and LOQ were statistically calculated using the data obtained by spiking each matrix (milk, egg, animal tissues, and corn and sorghum raw agricultural commodities) with 0.02 mg kg^{-1} propachlor equivalents. The method's LOD and LOQ for the NIPA were 0.005 and 0.015 mg kg^{-1}, respectively, for both crop and animal tissues. Some fortified matrices had acceptable recoveries at levels below the LOQ. The LLMV was 0.01 and 0.02 mg kg^{-1} for crop and animal commodities, respectively. The LLMV is defined as the lowest fortification level at which acceptable NIPA recovery and precision were demonstrated.

6 Multi-residue analytical method for the determination of acetochlor, alachlor, and metolachlor in aqueous samples

Identification/properties

Acetochlor	
Chemical name (IUPAC)	2-Chloro-*N*-ethoxymethyl-6′-ethylacet-*o*-toluidide
Structural formula	
Empirical formula	$C_{14}H_{20}ClNO_2$
Molecular mass	269.77
Melting point	10.6 °C
Boiling point	172 °C
Vapor pressure	0.0046 Pa at 25 °C
Solubility	Water 233 mg L^{-1} (25 °C) Miscible with organic solvents
Alachlor	
Chemical name (IUPAC)	2-Chloro-2′,6′-diethyl-*N*-methoxymethylacetanilide
Structural formula	
Empirical formula	$C_{14}H_{20}ClNO_2$
Molecular mass	269.77

Melting point	41 °C
Boiling point	>400 °C
Vapor pressure	0.0055 Pa at 25 °C
Solubility	Water 188 mg L^{-1} (pH 5, 20 °C), 170 mg L^{-1} (pH 7, 20 °C) Methanol >803 g L^{-1} (20 °C) 1,2-Dichloroethane >749 g L^{-1} (20 °C) n-Heptane 130 g L^{-1} (20 °C)
Metolachlor	
Chemical name (IUPAC)	2-Chloro-6′-ethyl-*N*-(2-methoxy-1-methylethyl)acet-*o*-toluidide
Structural formula	
Empirical formula	$C_{15}H_{22}ClNO_2$
Molecular mass	283.8
Vapor pressure	0.0009 Pa at 25 °C
Solubility	Water 488 mg L^{-1} (25 °C)

6.1 Outline of method

DropBooks

Acetochlor, alachlor, and metolachlor are determined in ground and surface water samples. Deuterated internal standards are added to each water sample, and analytes are extracted using an SPE column. After elution and concentration to an appropriate volume, the analytes are quantitated by GC/MS.

6.2 Apparatus

Mettler electronic analytical balance
12-port vacuum manifold for SPE columns with transparent sidewalls and pressure gauge
SPE reservoir, 75-mL, with and without frit
SPE adapter, 8-mL
Graduated cylinder, 250-mL
Octadecyl (C_{18}) SPE disposable extraction column, 2.8-mL capacity, 500-mg sorbent weight
Silica SPE disposable extraction column
Sea sand, washed
Volumetric flask, 100-mL
Disposable culture tubes, 100 × 13-mm i.d.
Volumetric glass pipets, various sizes
Pasteur pipets, 5.75- and 9-in lengths

DuPont/Sorvall RC-5B refrigerated centrifuge
Nalgene centrifuge bottle, 250-mL
Glass screw-cap autosampler vial, 2-mL, with Teflon-lined septum
Fisons MD-800 gas chromatograph/mass spectrometer or equivalent
MassLynx Software, Version 2
Restek Rtx-1MS fused-silica open-tubular column (FSOT) with integral guard column, 30 m × 0.25-mm i.d. and 0.25-μm film thickness
Eppendorf fixed-volume pipets, 0.50-mL
Eppendorf fixed-volume pipets, 1.0-mL
Eppendorf pipet tips, 1.0-mL
Amber-glass bottles with Teflon-lined caps, 4-oz

6.3 Reagents

The following standards may be available through the Environmental Protection Agency, National Pesticide Standard Repository, Fort Meade, MD, USA.

Acetochlor, analytical grade, ≥95% purity
Alachlor, analytical grade, ≥95% purity
Metolachlor, analytical grade, ≥95% purity

The following standards were synthesized specifically for this purpose.

Deuterated acetochlor, 2-chloro-*N*-(2-(2′-trideutero)ethyl-6-methylphenyl)-*N*-ethoxymethylacetamide, analytical grade, ≥92% purity
Deuterated alachlor, 2-chloro-*N*-(2,6-pentadeuterodiethyl-3,4,5-deuterophenyl)-*N*-methoxymethylacetamide, analytical grade, ≥95% purity
Deuterated metolachlor, 2-chloro-*N*-(2-(2′-trideutero)ethyl-6-methylphenyl)-*N*-(2-methoxypropan-2-yl)acetamide, analytical grade, ≥95% purity

The following reagents or their equivalents may be obtained from laboratory suppliers.

Ethyl acetate (EtOAc), Optima Grade, Fisher No. E196
2,2,4-Trimethylpentane (isooctane), Optima Grade, Fisher No. O301
Methanol (MeOH), Optima Grade, Fisher No. A454
Water, Optima Grade, Fisher No. W7-4
Ethanol (EtOH), absolute-200 proof, Midwest Grain Products No. 6810-00-242-3645
Sodium sulfate, anhydrous, Certified ACS, Fisher No. S421-500
Deionized water from a Milli-Q water purification system (Millipore Co.)

6.3.1 Reagent preparation

Prepare isooctane–ethyl acetate (9 : 1, v/v), water–methanol (4 : 1, v/v), and pre-washed sodium sulfate (combine 1500 g of sodium sulfate and 1.0 L of isooctane–ethyl acetate (9 : 1, v/v), and agitate the combination for 15 min). Filter and wash the sodium sulfate with approximately 250 mL of fresh solvent under vacuum.

6.4 Analytical standards

Analytical standards are prepared for two purposes: for fortifying control matrices to determine the analytical accuracy and for calibrating the response of the analyte in the mass spectrometer detector. The purity of all standards must be verified before preparation of the stock solutions. All standards should be refrigerated (2–10 °C) in clean amber-glass bottles with foil/Teflon-lined screw-caps. The absolute volume of the standard solutions may be varied at the discretion of the analyst, as long as the correct proportions of the solute and solvent are maintained. Calibrate the analytical balance before weighing any analytical standard material for this method.

6.4.1 Stock standard solutions

Prepare a 1000 $\mu g\,mL^{-1}$ individual herbicide solution by weighing 0.1000 ± 0.0010 g (weight adjusted for purity) of each analytical-grade herbicide into individual 100-mL volumetric flasks, dilute the contents to volume with absolute ethanol, and mix the solution to ensure complete dissolution. Mix and dilute individual herbicide solutions to prepare a 100.0 $\mu g\,mL^{-1}$ mixed herbicide solution (for extended standards), a 10.0 $\mu g\,mL^{-1}$ mixed herbicide solution, and a 1.00 $\mu g\,mL^{-1}$ mixed herbicide solution. The concentration values of the mixed herbicide solutions refer to each individual herbicide within the mixture.

For deuterated standards, prepare a 500 $\mu g\,mL^{-1}$ individual deuterated herbicide solution and a 10.0 $\mu g\,mL^{-1}$ mixed deuterated herbicide solution.

DropBooks

6.4.2 Fortification solutions

In order to estimate the analytical accuracy of the method with a given set of water samples, a certain number of control water samples should be fortified with a known amount of each herbicide. Control water samples are fortified at different analyte levels across the range of anticipated concentrations. For example, 0.010 g of analyte is necessary for a 0.05 $\mu g\,L^{-1}$ fortification of a 200-mL sample. This would be accomplished by adding 1.0 mL of a 0.010 $\mu g\,mL^{-1}$ solution to the sample. The deuterated standards are not incorporated in the fortification solutions but may be added to all control and fortified samples for internal correction of recovery. The following solutions are used to fortify control water samples:

1.00 $\mu g\,mL^{-1}$ of a mixed herbicide fortification solution
0.200 $\mu g\,mL^{-1}$ of a mixed herbicide fortification solution
0.020 $\mu g\,mL^{-1}$ of a mixed herbicide fortification solution
0.010 $\mu g\,mL^{-1}$ of a mixed herbicide fortification solution

The above solutions are adequate to fortify 200 mL of control water samples in the range 0.05–20.0 $\mu g\,L^{-1}$ of each analyte. Samples fortified at levels above 5.00 $\mu g\,L^{-1}$ are analyzed only with extended calibration standards.

6.4.3 Deuterated internal standard solution

To prepare 0.10 $\mu g\,mL^{-1}$ mixed deuterated herbicide internal standard solution, pipet 10.0 mL of the 10.0 $\mu g\,mL^{-1}$ mixed deuterated herbicide solution into a

Table 3 GC/MS calibration standards

Vol. of 1.00 μg mL^{-1} herbicide solution (mL)	Vol. of 10.0 μg mL^{-1} deuterated solution (mL)	Final volume (mL)	Final analyte concentration (μg L^{-1})
0.5	1.0	100	5.0
2.5	1.0	100	25.0
Vol. of 10.0 μg mL^{-1} herbicide solution (mL)	**Vol. of 10.0 μg mL^{-1} deuterated solution (mL)**	**Final volume (mL)**	**Final analyte concentration (μg L^{-1})**
1.0	1.0	100	100
2.0	1.0	100	200
4.0	1.0	100	400
7.0	1.0	100	700
Vol. of 100.0 μg mL^{-1} herbicide solution (mL)	**Vol. of 10.0 μg mL^{-1} deuterated solution (mL)**	**Final volume (mL)**	**Final analyte concentration (μg L^{-1})**
1.0	1.0	100	1000
Extended standards:			
Vol. of 100.0 μg mL^{-1} herbicide solution (mL)	**Vol. of 10.0 μg mL^{-1} deuterated solution (mL)**	**Final volume (mL)**	**Final analyte concentration (μg L^{-1})**
2.0	1.0	100	2000
3.0	1.0	100	3000
5.0	1.0	100	5000
10.0	1.0	100	10 000
20.0	1.0	100	20 000

1000-mL volumetric flask, dilute to volume with absolute ethanol, and mix the solution. This solution contains 0.10 μg mL^{-1} each of the five deuterated herbicides. Addition of 1.0 mL of this solution to each control and fortified water sample and each water specimen will result in 0.10 μg mL^{-1} each of the five deuterated herbicides in the final analytical sample when the volume is 1.0 mL.

6.4.4 Detector calibration standards

The detector calibration standards are made at convenient concentrations of each analyte. All standards should be refrigerated (2–10 °C) in clean amber-glass bottles with foil/Teflon-lined screw-caps. The absolute volume of the standard solutions may be varied at the discretion of the analyst, as long as the correct proportions of the solute and solvent are maintained.

Experience has shown a linear range from 5 to 1000 μg L^{-1} with the listed instrumentation. This is equivalent to 0.05–5.0 μg L^{-1} in a 200-mL water sample. The extended standards are only prepared and used when the analytes exceed approximately 6.00 μg L^{-1} (120% of 5 μg L^{-1}).

The following is an example of GC/MS calibration standard levels. Concentrations other than those shown below may also be prepared and used if necessary. The GC/MS calibration standards may be prepared as set out in Table 3.

Dilute each of the detector calibration standards to a final volume of 100 mL with isooctane–ethyl acetate (9 : 1, v/v). The final concentration of each deuterated component is 100 $\mu g\,L^{-1}$ in all analytical standards.

6.5 Analytical procedure

The following is a general method for ground and surface water samples. Interferences in particular samples may require modification of this method. The analytical sample size is 200 mL, but the volume may be varied depending on the concentration of analytes in the sample.

6.5.1 Aqueous sample preparation

Samples are generally prepared and analyzed in sets of 24, which include at least one control and one fortified control water sample.

The aqueous samples are removed from chilled storage, each is thoroughly mixed, and gross particulates are allowed to settle before removing the analytical aliquot. Depending on the appearance of the samples, begin with one of the following two paragraphs.

If the samples have a large amount of particulate matter, transfer approximately 220 mL into a 250-mL centrifuge bottle, and centrifuge the contents for 10 min at 11 000 rpm. If the samples are clear or just cloudy after centrifugation, transfer 200 mL of the sample into a 250-mL graduated cylinder (or other suitable container).

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Fortification of control water samples must be made at this point by adding the correct volume of the appropriate fortification solution. Optima Grade bottled water is used as the matrix for the controls and the laboratory-fortified samples for all water types. Add 1.0 mL of the 0.10 $\mu g\,mL^{-1}$ mixed deuterated herbicide internal standard solution to all samples including control and fortified water samples. A 200-mL volume is sufficient to quantitate to levels of 0.05 $\mu g\,L^{-1}$.

6.5.2 Solid-phase extraction

Place the C_{18} SPE column on the vacuum manifold, and prepare for extraction by washing the column sequentially three times with approximately 3-mL volumes of methanol and Optima water, respectively. Following the final water wash, allow a volume of liquid to remain on top of the resin bed, and avoid allowing the column to become dry before sample addition.

If the initial water samples were clear, skip the next paragraph, and continue.

If the initial water samples had a large amount of particulate matter or were cloudy, remove the C_{18} column, and place a 75-mL fritted reservoir on the vacuum manifold. Add approximately 10 g of sea sand to the reservoir. Wash the sea sand with approximately 20-mL volumes of isooctane–ethyl acetate (9 : 1, v/v), methanol, and Optima water, respectively. Following the final water wash, allow a small volume of liquid to remain on top of the sea-sand bed. Place the washed C_{18} column on

the manifold, and piggy-back the reservoir containing the washed sea-sand to the column.

Add a 75-mL reservoir with or without a frit to the top of the C_{18} column. Transfer the sample quantitatively to the reservoir. With a small vacuum applied to the chamber, draw the sample through the C_{18} column at a flow rate not exceeding 10 mL min^{-1}. Discard the sample eluent from the C_{18} column. When the entire sample has eluted through the C_{18} column, rinse the graduated cylinder with approximately 10 mL of Optima water, and add this rinsate to the reservoir. After the entire sample and rinse volume have eluted through the C_{18} column, remove the 75-mL reservoir, and wash the C_{18} column with 2 × approximately 2 mL of water–methanol (4 : 1, v/v). The wash step has two advantages: (1) to facilitate drying of the SPE cartridge and (2) partial removal of humic acids (or other high-boiling components) from the SPE before chromatography. Place a silica column on the C_{18} column, and dry the column under vacuum for approximately 20 min to remove all the water. The silica serves to prevent possible atmospheric contamination of the C_{18} column. Remove and retain the silica column. Piggy-back the C_{18} column to an 8-mL reservoir containing approximately 2 g of pre-washed sodium sulfate. Return the C_{18} column to its original position on the vacuum manifold. Dry the inside manifold tips with a paper towel, taking precautions against cross-contamination. Place a calibrated disposable culture tube in the rack below the C_{18} column. Add 1.0 mL of the isooctane–ethyl acetate (9 : 1, v/v) solution, and mark the meniscus to calibrate culture tubes. Add 2 × 2.5 mL of isooctane–ethyl acetate (9 : 1, v/v) to the C_{18} column. Slowly apply vacuum to the manifold, and elute the solvent at a rate of approximately 2–4 mL min^{-1}. The silica column may be replaced on top of the C_{18} column.

Inspect the culture tubes in the manifold to determine if there is water in the organic eluent for any sample. If a water layer is present, quantitatively transfer the organic phase into a clean culture tube using a small amount of additional solvent as necessary. Return the culture tube containing the organic extract to its proper location in the manifold rack. Remove the C_{18} and sodium sulfate tubes, and reinstall the silica tubes on the manifold. With the sample remaining in the culture tube, continue to apply vacuum to the manifold to remove excess solvent. When the solvent volume is <1 mL, discontinue vacuum, and allow the sample to return to room temperature. Adjust the sample volume in the culture tube to 1 mL with isooctane–ethyl acetate (9 : 1, v/v). Transfer the entire sample into an autosampler vial for GC/MS analysis. Sample extracts may be stored for up to 1 month in a refrigerator (<10 °C) before analysis.

6.5.3 Determination by GC/MS

(1) Detector calibration. A calibration curve should be generated for every set of samples with a minimum of five standards. The order of standards is nonsystematic throughout the set. The first and last sample in each analytical sample set must be a standard. Typically, each set will have several samples between the standard levels. The calibration curve is generated by plotting the ratio of the peak areas of each analyte and its deuterated analog against the concentration of each calibration standard. Least-squares estimates of the data points are used to define the calibration curve. Linear, exponential, or quadratic calibration curves may be used, but the analyte levels for all the samples from the same protocol must be analyzed with the same curve fit. In the event that analyte responses exceed the calibration range by more

Table 4 Typical quantification and confirmation ions

Analyte	Quantification ion (Da)	Confirmation ion (Da)
Acetochlor	162	146
d_3-Acetochlor	165	149
Alachlor	188	160
d_{13}-Alachlor	200	171
Metolachlor	162	240
d_3-Metolachlor	165	243

than approximately 20%, the samples must be reanalyzed with the extended standards. This reanalysis necessitates a reduction in instrument sensitivity that may be accomplished by reducing the electron multiplier voltage or by using another instrument with lower sensitivity.

(2) GC/MS. GC/MS is used for separation and quantification of the herbicides. Data acquisition is effected with a data system that provides complete instrument control of the mass spectrometer. The instrument is tuned and mass calibrated in the EI mode. Typically, four ions are monitored for each analyte (two ions for each herbicide and two ions for the deuterated analog). If there are interferences with the quantification ion, the confirmation ion may be used for quantification purposes. The typical quantification and confirmation ions for the analytes are shown in Table 4. Alternative ions may be used if they provide better data.

The gas chromatograph is operated under the following conditions. The conditions may require modification to achieve satisfactory sensitivity and separation.

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(3) Operating conditions

Column	Restek Rtx-1MS FSOT with integral guard column, 30 m × 0.25-mm i.d., 0.25-μm film thickness
Gas	Helium
Flow rate	0.7–1.0 mL min^{-1}
Injection volume	1–5 μL
Injection mode	Splitless
Injector temperature	240 °C
Ion source temperature	200 °C
Interface temperature	270 °C
Column temperature	100–185 °C at 20 °C min^{-1} 185–195 °C at 1 °C min^{-1} 195–300 °C at 30 °C min^{-1} 300 °C held for 8 min
Total run time	~30 min, injection to injection

(4) Column/injector maintenance. The use of a guard column, in addition to scheduled injector maintenance, provides significantly improved GC performance. Loss of performance manifests itself in poor peak shape and reduced sensitivity. This is particularly noticeable with atrazine but also with the other analytes. Maintenance should include removal of a 30–60 cm section of the guard column and replacement of the

injection port liner and associated seals and O-rings. In the HP 5890 gas chromatograph, this would include replacement of the gold-plated metal seal at the base of the injector. The frequency of this scheduled maintenance is based on the quality and number of samples analyzed and the performance of the method.

6.6 Calculation of residues

Linear, exponential, or quadratic calibration curves may be used to quantitate the amount of analyte in each sample. Quantitation of each analyte is made independently.

6.6.1 Quantitation using a linear calibration curve

The concentration of the analyte in the injected sample is determined based on the height or area of the analyte peak and interpolation of the internal or external standard linear calibration curve according to the following equation:

$$(\mu g\, L^{-1}\ \text{analyte})\, m + b = PKR_{\text{analyte}}$$

where PKR_{analyte} is the detector response (as peak height, area, or ratio of the natural isotope over the deuterated analog, i.e., m/z 162/165) of the analyte, m is the slope of the linear least squares fit of the calibration curve, and b is the y-intercept of the linear least-squares fit of the calibration curve.

The resulting concentration value in micrograms per liter represents the concentration of the analyte in the injected sample. Using the concentration of analyte in the injected sample, the final extract volume, and the volume of water extracted, the concentration of analyte present in the water specimen can be calculated. The concentration ($\mu g\, L^{-1}$) of herbicide in the water specimen is calculated by multiplying the analyte concentration ($\mu g\, L^{-1}$) by the final volume (mL) and dividing by the water specimen volume (mL):

$$\text{ppb (herbicide)} = \frac{(\mu g\, L^{-1}\text{herbicide found})(\text{final volume in mL})}{\text{water specimen volume in mL}}$$

6.6.2 Quantitation using an exponential calibration curve

The concentration of the analyte in the injected sample is determined based on the height or area of the analyte peak and interpolation of the internal or external standard exponential calibration curve according to the following equation:

$$\ln(PKR_{\text{analyte}}) = A + B \ln(\mu g\, L^{-1}\text{analyte})$$

Thus,

$$\ln(\mu g\, L^{-1}\text{analyte}) = [\ln(PKR_{\text{analyte}}) - A]/B$$
$$\ln(\mu g\, L^{-1}\text{analyte}) = (-A/B) + (1/B)\ln(PKR_{\text{analyte}})$$

where PKR_{analyte} is the detector response (as peak height, area, or ratio of the natural isotope over the deuterated analog, i.e., m/z 162/165) of the analyte, A is the

y-intercept of the curve of the natural logarithm of the concentration and detector response, and B is the slope of the curve of the natural logarithm of the concentration and detector response.

Once the concentration of the analyte in micrograms per liter has been determined in the injected sample, the remainder of the calculations is the same as for the linear calibration curve.

6.6.3 Quantitation using a quadratic calibration curve

The concentration of the analyte in the injected sample is determined based on the height or area of the analyte peak and interpolation of the internal or external standard quadratic calibration curve according to the following equation:

$$A(\mu g\,L^{-1}\ \text{analyte})^2 + B(\mu g\,L^{-1}\ \text{analyte}) + C = PKR_{\text{analyte}}$$

where PKR_{analyte} is the detector response (as peak height, area, or ratio of the natural isotope over the deuterated analog, i.e., m/z 162/165) of the analyte, and A, B, and C are curve constants.

Once the concentration of the analyte in micrograms per liter has been determined in the injected sample, the remainder of the calculations is the same as for the linear calibration curve.

6.7 Evaluation

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6.7.1 Modifications or potential problems

Low-level interferences are present in ground- and surface water samples. The water–methanol (4 : 1, v/v) wash in the SPE phase of the sample workup is intended to minimize these interferences while maintaining quantitative recovery of the analytes. A solvent blank may be injected with the samples as part of an analytical set to confirm the cleanliness of a solvent used.

Initially, this method utilized 5-mL conical centrifuge tubes as the collection device for final elution of the extract from the C_{18} tubes. In practice, these tubes were found to be very difficult to clean and in few instances were the cause of cross-contamination when low-concentration samples were extracted following samples with very high concentrations. Since no commercial graduated tubes were available, disposable culture tubes are used as the receiver. These tubes are individually calibrated before use. A solvent blank sample may be processed through the method from extraction to quantification to determine if contamination from glassware occurs.

6.7.2 Analytical accuracy, limits of detection and quantitation

(1) Analytical accuracy. The mixture of all deuterium-labeled internal standards is added to each water sample before extraction. This does not prevent the loss of the unlabeled herbicides from the sample in subsequent processing steps, but a proportional loss of the deuterated internal standard precludes the need to correct for recovery. Although referring to recovery in this type of analysis is inappropriate, the accuracy of this method should be monitored.

The estimated analytical accuracy of the method can be obtained from the mean of the accuracies of each individual fortification using the following equation:

$$\text{estimated accuracy} = \frac{\sum C_f / A_f(100\%)}{N_i}$$

where C_f is the concentration of herbicide found in the fortified control sample, A_f is the concentration fortified into the control sample, and N_i is the number of fortified control samples.

There should be nearly equal numbers of fortifications at each level, so the estimated analytical accuracy will not be disproportionately weighted.

If a control water sample to be fortified is found to contain a significant concentration of any of the five herbicides, then this concentration is subtracted from the amount found in the fortified control sample in order to calculate the accuracy for the sample. This is done for those samples that have been found to contain low concentrations with respect to the fortification level. As a rule, the amount fortified should be at least twice the concentration found in the identical sample that is not fortified. In this case, the variable C_f in the above equation should be replaced by the expression $(C_f - C_b)$, where C_b is the concentration found in the identical sample that was not fortified.

6.7.3 Limits of detection and quantitation

Limits of detection for each of the three parent herbicides in surface and groundwater were determined using results obtained from control samples analyzed along with hundreds of surface and ground water sets during the years 1995–2001. In each of these years, the calculated LODs (minimum detectable true concentrations/detection) were below 0.03 $\mu g\,L^{-1}$ for acetochlor and metolachlor and 0.05 $\mu g\,L^{-1}$ for alachlor. A detection criterion is a measured concentration threshold that defines a likely upper bound for samples not containing the analyte. If the actual concentration of an analyte is at this detection limit or greater, there is at least a 95% chance of detection.

LOQs for each of the three parent herbicides in surface water were determined using all the analytical results (not corrected for background) of samples fortified at the lowest fortification level, 0.05 $\mu g\,L^{-1}$, during the analysis in years 1995–2001. The calculated LOQs were below 0.05 $\mu g\,L^{-1}$ for acetochlor and metolachlor and approximately 0.05 $\mu g\,L^{-1}$ for alachlor. If the true concentration of an analyte is at the LOQ or greater, the standard error of individual measured concentration values relative to the true concentration is at most $\pm 10\%$.

This analytical method provides very good precision and accuracy for the three parent herbicides over a 0.05–5.00 $\mu g\,L^{-1}$ range. Validation has been extended up to 20 $\mu g\,L^{-1}$.

7 Multi-residue analytical method for the determination of acetochlor, alachlor, and metolachlor soil metabolites in aqueous samples

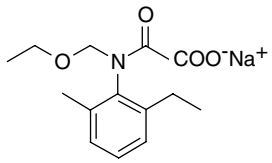
An analytical method for the following soil degradates was developed.

Identification/properties

Acetochlor oxanilic acid

Chemical name (IUPAC) *N*-Ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) oxamic acid, sodium salt

Structural formula



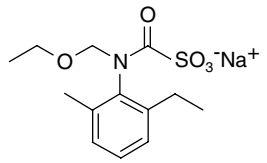
Empirical formula $C_{14}H_{18}NO_4Na$

Molecular mass 287.3

Acetochlor sulfonic acid

Chemical name (IUPAC) [Ethoxymethyl-(2-ethyl-6-methylphenyl)carbamoyl] methanesulfonic acid, sodium salt

Structural formula



Empirical formula $C_{14}H_{20}NO_5SNa$

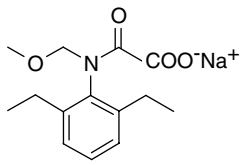
Molecular mass 337.4

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Alachlor oxanilic acid

Chemical name (IUPAC) *N*-(2,6-Diethylphenyl)-*N*-methoxymethyloxamic acid, sodium salt

Structural formula



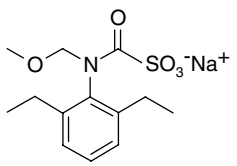
Empirical formula $C_{14}H_{18}NO_4Na$

Molecular mass 287.3

Alachlor sulfonic acid

Chemical name (IUPAC) [(2,6-Diethylphenyl)methoxymethylcarbamoyl] methanesulfonic acid, sodium salt

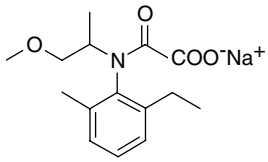
Structural formula



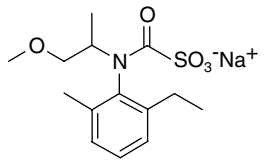
Empirical formula $C_{14}H_{20}NO_5SNa$

Molecular mass 337.4

Metolachlor oxanilic acid

Chemical name (IUPAC)	*N*-(2-Ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)oxamic acid, sodium salt
Structural formula	
Empirical formula	$C_{15}H_{20}NO_4Na$
Molecular mass	301.32

Metolachlor sulfonic acid

Chemical name (IUPAC)	[(2-Ethyl-6-methylphenyl)-(2-methoxy-1-methylethyl) carbamoyl]methanesulfonic acid, sodium salt
Structural formula	
Empirical formula	$C_{15}H_{22}NO_5SNa$
Molecular mass	351.4

7.1 Outline of method

This analytical method determines levels of major oxanilate and sulfonate soil metabolites of acetochlor, alachlor, and metolachlor in groundwater and surface water. The method consists of analysis of environmental samples by direct aqueous injection reversed-phase LC/MS/MS.

7.2 Apparatus

Electronic analytical balance
Volumetric flasks 100- to 1000-mL
Volumetric glass pipets, various sizes
Pasteur pipets, 5.75- and 9-in lengths
Refrigerated centrifuge
Nalgene centrifuge bottle, 250-mL
Disposable syringes, 5-cm^3
Disposable syringe filters, 25-mm × 0.45-mm
Glass autosampler vial (2-mL) with Teflon-lined septum
PE Sciex API 3000 MS/MS system using Sciex Analyst software
Hewlett-Packard Model 1100 HPLC system, including G1312A binary pump, G1322A degasser, G1313A autosampler, and G1316A column heater
Analytical column, Betasil C_{18}, 100 mm × 2-mm i.d., 5-mm film thickness

Guard column, Betasil C_{18}, 10 mm × 2-mm i.d.
Guard column holder
Eppendorf fixed-volume pipets, 0.50-mL
Eppendorf fixed-volume pipets, 1.0-mL
Eppendorf pipet tips, 1.0-mL

7.3 Reagents

The following reagents may be available through the Environmental Protection Agency, National Pesticide Standard Repository, Fort Meade, MD, USA:
Acetochlor oxanilic acid, sodium salt: analytical grade, ≥95% purity
Acetochlor sulfonic acid, sodium salt: analytical grade, ≥95% purity
Alachlor oxanilic acid, sodium salt: analytical grade, ≥95% purity
Alachlor sulfonic acid, sodium salt: analytical grade, ≥90% purity
Metolachlor oxanilic acid, sodium salt: analytical grade, ≥95% purity
Metolachlor sulfonic acid, sodium salt: analytical grade, ≥95% purity

The following reagents are available from laboratory suppliers:

Methanol, MeOH: Optima grade
Reagent water: Optima grade
Ammonium acetate: ACS reagent grade
Acetonitrile (ACN): EM Omnisolve HPLC grade or equivalent
Formic acid: EM reagent EM-FX0440-11 or equivalent
Ethanol, EtOH (absolute-200 proof)
Deionized water from a Milli-Q water purification system (Millipore Co.).

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7.3.1 Reagent preparation

Prepare an adequate quantity of the following solutions and reagents:

1 M ammonium acetate (NH_4OAc): dissolve 77.09 g of NH_4OAc in 1 L of deionized H_2O.
50 mM NH_4OAc: combine 50 mL of 1 M NH_4OAc and 950 mL of deionized H_2O.
Mobile phase A (5 mM NH_4OAc with approximately 0.1% formic acid): combine 100 mL of 50 mM NH_4OAc, 900 mL of deionized H_2O, and 1 mL of formic acid.
Mobile phase B [ACN–50 mM NH_4OAc, 9:1 (v/v), approximately 0.1% formic acid): combine 900 mL of ACN, 100 mL of 50 mM NH_4OAc, and 1 mL of formic acid.

7.4 Analytical standards

Analytical standards are prepared for two purposes: for fortifying control matrices to determine the analytical accuracy and for calibrating the response of the analyte in the mass spectrometer. The purity of all standards must be verified before preparation of the stock solutions. All standard solutions (stock, fortification, and calibration) should be stored refrigerated (2–10 °C) in clean amber-glass bottles with foil/

Teflon-lined screw-caps. The absolute volume of the standard solutions may be varied at the discretion of the analyst, as long as the correct proportions of the solute and solvent are maintained. The stock solutions below are adequate to prepare fortification and calibration standards in the range 0.10–20.0 $\mu g\,L^{-1}$ of each analyte. Calibrate the analytical balance before weighing any neat analytical standard for this method.

7.4.1 Stock standard solutions

To prepare 1000 $\mu g\,mL^{-1}$ individual metabolite solutions, weigh 0.1000 ± 0.0010 g (weight adjusted for purity of free acid) of each analytical-grade metabolite into individual 100-mL volumetric flasks. Dilute the contents to volume with absolute ethanol, and mix the contents to ensure complete dissolution. Dilute equal amounts of each metabolite solution to the appropriate volume with reagent water to prepare a 1.0 $\mu g\,mL^{-1}$ mixed metabolite solution, a 100.0 $\mu g\,L^{-1}$ mixed metabolite solution, and a 10.0 $\mu g\,L^{-1}$ mixed metabolite solution. The concentration values of the mixed metabolite solutions refer to each individual metabolite within the mixture.

7.4.2 Fortification solutions

In order to estimate the analytical accuracy of the method within a given set of water samples, a number of control water samples should be fortified with a known amount of each metabolite. Control water samples are fortified at different analyte levels across the range of anticipated concentrations. The aqueous solutions used to fortify control water samples are prepared at 0.10, 0.25, 0.50, 1.0, 2.0, 5.0, 10.0, and 20 $\mu g\,L^{-1}$.

7.4.3 Detector calibration standards

The detector calibration standards are made at convenient concentrations of each analyte. Experience has shown that linearity can be obtained over a range from 0.25 to 20.0 $\mu g\,L^{-1}$ with the listed instrumentation. Concentrations other than those shown below also may be prepared and used if necessary. The LC/MS/MS calibration standards are prepared at 0.10, 0.25, 0.50, 1.0, 2.0, 5.0, 10.0, and 20.0 $\mu g\,L^{-1}$.

7.5 Analytical procedures

The following is a general method for groundwater and surface water samples. Unique interferences in particular samples may require modification of this method. If modifications are necessary, they should be fully documented in the raw data.

7.5.1 Aqueous sample preparation

Samples are generally prepared and analyzed in sets of 30 that include at least one control and one fortified control water sample. Optima-grade bottled water may be used as the matrix for the controls and the laboratory-fortified samples for all water types. Depending on the appearance of the samples, filtration may be required.

Groundwater and raw surface water are typically filtered through a 0.45-μm filter before analysis. This is not generally required of finished surface water.

Approximately 1–2 mL of the sample is transferred directly into an autosampler vial for LC/MS/MS analysis.

7.5.2 Determination by LC/MS/MS

(1) Detector calibration. A calibration curve is generated for every set of samples with a minimum of five standard levels. The standards are interspersed among the analytical samples of each set. The first and last sample in each analytical sample set must be a standard.

The calibration curve is generated by plotting the peak area of each analyte in a calibration standard against its concentration. Least-squares estimates of the data points are used to define the calibration curve. Linear, exponential, or quadratic calibration curves may be used, but the analyte levels for all the samples from the same protocol must be analyzed with the same curve fit. In the event that analyte responses exceed the upper range of the standard calibration curve by more than 20%, the samples must be reanalyzed with extended standards or diluted into the existing calibration range.

(2) LC/MS/MS. LC/MS/MS is used for separation and quantitation of the metabolites. Using multiple reaction monitoring (MRM) in the negative ion electrospray ionization (ESI) mode, LC/MS/MS gives superior specificity and sensitivity to conventional liquid chromatography/mass spectrometry (LC/MS) techniques. The improved specificity eliminates interferences typically found in LC/MS or liquid chromatography/ultraviolet (LC/UV) analyses. Data acquisition is accomplished with a data system that provides complete instrument control of the mass spectrometer.

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The instrument must be properly tuned and mass calibrated in the negative ion ESI mode. Typically, two ions are monitored for each analyte, one transition (parent) ion for each metabolite, and one quantitation (fragment) ion for each metabolite. The typical transition and quantitation ions for the analytes are shown in Table 5. Alternative ions may be used if they provide better data (sensitivity and/or specificity).

The following are suggested liquid chromatography (LC) instrument conditions. The conditions may require modification to achieve satisfactory sensitivity and resolution.

Table 5 Typical transaction and quantitation ions

Analyte	MRM transition ion (Da)	Quantitation ion (Da)
Acetochlor oxanilic acid	264	146
Alachlor oxanilic acid	264	160
Metolachlor oxanilic acid	278	206
Acetochlor sulfonic acid	314	162
Alachlor sulfonic acid	314	176
Metolachlor sulfonic acid	328	121

LC operating conditions	
LC pump	HP 1100 HPLC binary pump (or equivalent)
Autoinjector	HP 1100 (or equivalent)
Column	Betasil C_{18} (5-μm), 100 × 2-mm i.d.
Guard	Betasil C_{18} (5-μm), 10 × 2-mm i.d.
Mobile phase A	5 mM NH_4OAc with approximately 0.1% formic acid
Mobile phase B	ACN–50 mM NH_4OAc (9:1, v/v) with approximately 0.1% formic acid
Flow rate	0.3 mL min^{-1} [post-column split at approximately 2 : 1 (0.2 mL min^{-1} to ion source)]
Gradient (A:B)	Initial conditions: 90:10 Linear gradient: 30:70 in 7 min Re-equilibrate for approximately 3 min
Divert	Divert flow to waste for approximately 3 min after injection
Injection volume	100 μL

The MRM experiments do not require chromatographic separation of the metabolites. Therefore, other LC conditions, columns, gradient, and injection volumes may be used provided that there is adequate sensitivity and specificity, and the chromatographic quality is not compromised.

Typical ESI instrument parameters are as follows:

ESI conditions	
Ionization mode	Negative
Ionspray voltage	4.2 kV
Curtain gas	8 at approximately 80 psi (nitrogen)
Nebulizer pressure	12 at approximately 80 psi (nitrogen)
Turbo temperature	350 °C
Turbo gas flow	6 L min^{-1} (nitrogen)
Total run time	About 10 min, injection to injection

These conditions may be changed to obtain optimal instrument performance and to maximize sensitivity. The actual conditions used for sample analysis are recorded in the raw data.

(3) Column/injector maintenance. The use of a guard column provides significantly improved LC performance. Loss of performance manifests itself in poor peak shape and reduced sensitivity. Plugging of the guard column results in increased back-pressure on the pumping system. The frequency of this maintenance is at the discretion of the analyst and is based on the quality and number of samples analyzed and the historical performance of the method. The maintenance should be based on the peak performance and back-pressure rather than a fixed time interval. With the method as described, at least 1000 environmental samples have been analyzed without significant negative effects on performance.

7.6 Calculation of residues

Linear, exponential, or quadratic calibration curves may be used to quantitate the amount of analyte in each sample. Quantification of each analyte is made independently.

7.6.1 Quantitation using a linear calibration curve

The concentration of the analyte in the injected sample is determined based on the height or area of the analyte peak and interpolation of the internal or external standard linear calibration curve according to the following equation:

$$(\mu g\, L^{-1} \text{analyte})m + b = PKR_{\text{analyte}}$$

where PKR_{analyte} is the detector response (as peak height or area) of the analyte, m is the slope of the linear least-squares fit of the calibration curve, and b is the y-intercept of the linear least squares fit of the calibration curve.

Generally, no sample concentration or dilution is involved, and the resulting concentration value is taken directly from the regression curve and represents the concentration of the analyte in the injected sample. If dilution is necessary, then the ratio of the original and final volumes is included in the calculation as shown in the equation

$$\text{ppb (degradate)} = \frac{(\mu g\, L^{-1} \text{degradate found})(\text{final volume in mL})}{\text{water specimen volume in mL}}$$

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Weighting of the calibration curve, $1/x$ or $1/x^2$, is expected to provide better curve fit at the lower concentration levels. Alternative calculations, such as exponential or quadratic curve fits, are acceptable if they provide improved precision and/or accuracy.

7.7 Evaluation

7.7.1 Modifications or potential problems

At least one control water sample must be analyzed concurrently with the water samples to determine the presence of matrix interferences and/or background levels of the metabolites. Optima-grade bottled water is used as the matrix for the controls and the fortified samples for all water types, because obtaining ground and surface water specimens that are completely free of the metabolites is difficult. Our analyses of ground and surface waters have demonstrated the presence of low-level interferences in these matrices. Interferences from other pesticides are unknown, because none have been examined. However, none are expected due to the high level of specificity of the LC/MS/MS analysis.

A solvent blank may be injected with the samples as part of an analytical set to confirm the cleanliness of a solvent used.

Disposable labware should be used where possible.

Standard addition experiments can be used to check for matrix effects such as ion enhancement or suppression.

7.7.2 Analytical accuracy, limits of detection, and quantitation

Analytical accuracy. The estimated analytical accuracy of the method can be obtained from the mean of the accuracies of each individual fortification using the following equation:

$$\text{estimated accuracy} = \frac{\sum C_f / A_f(100\%)}{N_i}$$

where C_f is the concentration of metabolite found in the fortified control sample, A_f is the concentration fortified into the control sample, and N_i is the number of fortified control samples.

There should be nearly equal numbers of fortifications at each level, so the estimated analytical accuracy will not be disproportionately weighted. If a control water sample to be fortified is found to contain a significant concentration of any of the six metabolites, then this concentration is subtracted from the amount found in the fortified control sample in order to calculate the accuracy for the sample. This is done for those samples that have been found to contain low concentrations with respect to the fortification level.

Generally, the amount fortified should be at least twice the concentration found in the identical sample that is not fortified. In this case, the variable C_f in the above equation should be replaced by the expression $(C_f - C_b)$, where C_b is the concentration found in the identical sample that was not fortified.

7.7.3 Limits of detection and quantifiation

Limits of detection for each of the six soil metabolites in surface water and groundwater were determined by using an estimate of variability for the 0.25 $\mu g\,L^{-1}$ fortifications from samples analyzed along with hundreds of surface water and groundwater sets during the years 1999–2001. During these years, the estimated LODs were below 0.1 $\mu g\,L^{-1}$ for acetochlor sulfonic acid, acetochlor oxanilic acid, alachlor oxanilic acid, metolachlor sulfonic acid, and metolachlor oxanilic acid and about 0.1 $\mu g\,L^{-1}$ for alachlor sulfonic acid. If the actual concentration of an analyte is at this detection limit or greater, there is at least a 95% chance of detection.

LOQs for each of the six soil metabolites in surface water and groundwaters were determined using analytical results (not corrected for background) of samples fortified at the lowest fortification level, 0.25 $\mu g\,L^{-1}$, during the analysis in years 1999–2001. The calculated LOQs for acetochlor oxanilic acid, metolachlor sulfonic acid and metolachlor oxanilic acid are below 0.25 $\mu g\,L^{-1}$. The calculated LOQs for acetochlor sulfonic acid, alachlor sulfonic acid, and alachlor oxanilic acid are below 0.10 $\mu g\,L^{-1}$. If the true concentration of an analyte is at the LOQ or greater, the standard error of individual measured concentration values relative to the true concentration is at most $\pm 10\%$.

This analytical method provides very good precision and accuracy for the six soil metabolites over a 0.25–20.0 $\mu g\,L^{-1}$ range.

8 Future directions for environmental monitoring

Environmental monitoring of chloroacetanilides requires methods that have the capability to distinguish between complex arrays of related residues. The two example methods detailed here for water monitoring meet this requirement, but the method for metabolites requires sophisticated mass spectral equipment for the detection of directly injected water samples. In the near term, some laboratories may need to modify this method by incorporation of an extraction/concentration step, such as SPE, that would allow for concentration of the sample, so that a less sensitive and, correspondingly, less expensive, mass spectral detector can be used. However, laboratories may want to consider purchasing a sensitive instrument rather than spending time on additional wet chemistry procedures. In the future, sensitive instrumentation may be less expensive and available to all laboratories. Work is under way to expand the existing multi-residue methods to include determination of additional chloroacetanilides and their metabolites in both water and soil samples.

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References

1. M.J. Warner and D.D. Arras, 'Alachlor,' in "Comprehensive Analytical Profiles of Important Pesticides," ed. J. Sherma and T.C. Cairns, CRC Press, Boca Raton, FL, Chapter 5, pp.109–129 (1993).
2. R.L. Graves, 'Determination of nitrogen and phosphorus containing pesticides in water by gas chromatography with a nitrogen–phosphorus detector,' Method 507, in "Methods for the Determination of Organic Compounds in Drinking Water," Environmental Monitoring Systems Laboratory, US Environmental Protection Agency, Washington, DC (1991).
3. R.L. Graves, 'Determination of chlorinated pesticides in water by gas chromatography with an electron capture detector,' Method 508, in "Methods for the Determination of Organic Compounds in Drinking Water," Environmental Monitoring Systems Laboratory, US Environmental Protection Agency, Washington, DC (1991).
4. J.W. Eichelberger, J.W. Munch, and J.A. Shoemaker, 'Determination of organic compounds in drinking water by liquid–solid extraction and capillary column gas chromatography/mass spectrometry,' Method 525.2, in "Methods for the Determination of Organic Compounds in Drinking Water," Environmental Monitoring Systems Laboratory, US Environmental Protection Agency, Washington, DC (1994).
5. J.W. Worley, M.L. Rueppel, and F.L. Rupel, *Anal. Chem.*, **52**, 1845 (1980).
6. S.A. Schuette, R.G. Smith, L.R. Holden, and J.A. Graham, *Anal. Chim. Acta*, **236**, 141 (1990).
7. L.Q. Huang, *J. Assoc. Off. Anal. Chem.*, **72**, 349 (1989).
8. D.S. Aga, S. Heberle, D. Rentsch, R. Hany, and S.R. Mueller, *Environ. Sci. Technol.*, **33**, 3462 (1999).
9. C. Macomber, R.J. Bushway, L.B. Perkins, D. Baker, T.S. Fan, and B.S. Ferguson, *J. Agric. Food Chem.*, **40**, 1450 (1992).

10. J.A. Shoemaker, 'Analytical method development for alachlor ESA and other acetanilide herbicide degradation products,' Presented at the 49th ASMS Conference, Chicago, IL, May 27–31, 2001.
11. K.A. Hostetler and E.M. Thurman, 'Determination of Chloroacetanilide Herbicide Metabolites in Water Using High-performance Liquid Chromatography–Diode Array Detection and High-performance Liquid Chromatography/Mass Spectrometry,' in Water-Resources Investigation Report 99–4018B, ed. D.W. Morganwalp and H.T. Buxton, US Geological Survey, Reston, VA (1999).
12. I. Ferrer, E.M. Thurman, and D. Barcelo, *Anal. Chem.*, **69**, 4547 (1997).
13. J.D. Vargo, *Anal. Chem.*, **70**, 2699 (1998).
14. J.D. Vargo, 'Determination of metolachlor and environmental degradates in water and soil by LC/MS/MS,' Presented at the 219th ACS National Meeting, San Francisco, CA, March 26–30, 2000.
15. P.C. Feng, S.J. Wratten, E.W. Logusch, S.R. Horton, and R.C. Sharp, in 'ACS Symposium Series, Immunochemical Methods in Environmental Analysis,' American Chemical Society, Washington, DC, Chapter 15 (1990).
16. D.S. Aga and E.M. Thurman, *Anal. Chem.*, **65**, 2894 (1993).
17. M.L. Pommes, E.M. Thurman, and D.A. Goolsby, in 'ACS Symposium Series, Immunochemical Methods in Environmental Analysis,' American Chemical Society, Washington, DC, Chapter 16 (1996).
18. S.M. Schraer, D.R. Shaw, M.Boyette, R.H. Coupe, and E.M. Thurman, *J. Agric. Food Chem.*, **48**, 5881 (2000).
19. P. Casino, S. Morais, R. Puchdes, and A. Maquieira, *Environ. Sci. Technol.*, **35**, 4111 (2001).
20. A.G. Hackett, 'Chloroacetanilides' in "Metabolic Pathways of Agrochemicals Part 1: Herbicides and Plant Growth Regulators," ed. T. Roberts, Royal Society of Chemistry, Cambridge, pp. 179–218 (1998).
21. A.G. Hackett, J.D. Fuhrman, and C.T. Stone, 'Acetochlor surface-water monitoring program: results to date,' Presented at the 218th ACS National Meeting, New Orleans, LA, August 22–26, 1999.
22. P. Hendley, J.D. Fuhrman, N.P. de Guzman, and W.E. Teskey, 'Acetochlor state groundwater monitoring program: results to date,' Presented at the 218th ACS National Meeting, New Orleans, LA, August 22–26, 1999.
23. N.P. de Guzman, 'State Ground Water Monitoring Program for Acetochlor and Other Corn Herbicides–Part 7. 2000 Progress Report,' Acetochlor Registration Partnership Report ARP-GWM-00, Acetochlor Registration Partnership (Monsanto, St. Louis, MO; Zeneca Ag Products, Wilmington, DE) (2001).

Dinitroaniline herbicides

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1 Introduction

Benfluralin, ethalfluralin, oryzalin, pendimethalin, prodiamine, trifluralin, etc., are dinitroaniline herbicides used for selective weed control in agronomic and horticultural applications (Figure 1). These compounds are used as soil-incorporated herbicides for pre-emergence control of annual grass and broad-leaved weed on cropland, lawns, and nonagricultural land. Germinating plants absorb dinitroaniline herbicides through the young foliage and roots from the soil layer where the applied herbicides are present 1–2 cm beneath the soil surface. The absorbed chemicals transport to the meristem, which act to impair cell division.

Dinitroaniline herbicides, in general, are very lipophilic, hence they are insoluble in water. They are stable under acidic or alkaline conditions. Dinitroaniline herbicides are potentially dissipated in the environment via photodegration and volatilization.

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Owing to its low water solubility and high octanol/water partition coefficients, dinitroaniline herbicides adsorb and bind to soil macromolecules and show minimal leaching potential. Dinitroanilines herbicides show good soil residue activities with soil half-lives ranging from 30 days for benfluralin and oryzalin to 6–7 months for trifluralin.[1] *N*-Dealkylation (aerobic conditions) and reduction of the nitro group to an amino moiety (anaerobic conditions) have been reported as major soil degradation pathways.

Dinitroaniline herbicides show minimal plant systematic translocation properties with the majority of the absorbed residues in the root tissues. Metabolites identified include traces of *N*-dealkylation, alkyl and aryl hydroxylation and nitro reduction products. Low levels of dinitroaniline herbicide residues have been reported in raw agricultural commodities according to Good Agricultural Practice.

The tolerance for pesticide residues (TPR) in Japan of pendimethalin is 0.2 mg kg^{-1} for cereals such as rice grain, wheat and corn; 0.05–0.2 mg kg^{-1} for beans such as red bean, soybean and peanut; 0.05–0.1 mg kg^{-1} for fruits such as peach, orange, apple, banana, papaya, strawberry and grape; 0.05–0.2 mg kg^{-1} for vegetables such as cabbage, tomato, eggplant, carrot, sugar beat and onion; 0.05 mg kg^{-1} for nuts such as almond, chestnut and walnut; and 0.05–0.2 mg kg^{-1} for potatoes. TPRs for trifluralin are 0.05–0.1 mg kg^{-1} for cereals, 0.05–0.15 mg kg^{-1} for beans, 0.05 mg kg^{-1}

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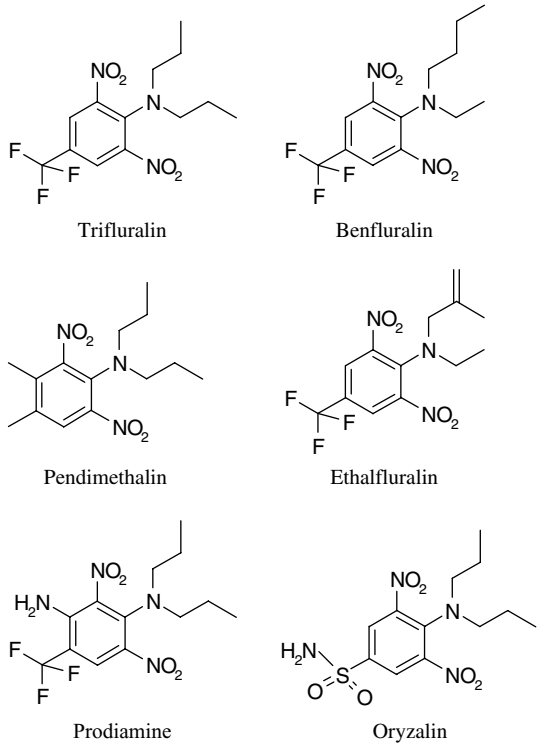


Figure 1 Structures of dinitroaniline herbicides

for fruits, 0.05–3 mg kg^{-1} for vegetables, 2.0 mg kg^{-1} for nuts, 0.05–0.15 mg kg^{-1} for potatoes, and 15 mg kg^{-1} for tea.[2]

Residue analytical methods for dinitroaniline herbicides in crops, soil, and water samples have been developed. The basic principle of the method consists of the following steps: extraction from the samples with acetone or other organic solvents, purification using liquid–liquid partition and column chromatography, and quantitative analysis by gas chromatography/nitrogen–phosphorus detection (GC/NPD) or gas chromatography/electron capture detection (GC/ECD). Column chromatography is used as the primary cleanup step to simplify the procedure and to enhance accuracy and sensitivity for the residue method.

2 Analytical methodology for plant materials

2.1 Nature of the residues

Owing to the potential for low levels of residues (parent plus metabolites) in crop tissues, the definition of dinitroaniline residues in crop samples is expressed as the parent molecule only.

2.2 Method principle

A homogenized sample of cereals, vegetables, fruits or potatoes (10–20 g) is extracted with an organic solvent such as acetone and methanol. After filtration, the extract is concentrated to about 20 mL by rotary evaporation below 40 °C. The residue is transferred with 5% sodium chloride (NaCl) aqueous solution and partitioned twice with n-hexane. The n-hexane extracts are dried with anhydrous sodium sulfate and subjected to a Florisil column chromatographic cleanup procedure. The eluate from the Florisil column is concentrated to dryness and the residue is dissolved in an appropriate amount of acetone for analysis by GC/NPD.[3]

2.2.1 Extraction

(1) Vegetables and fruits

A 20-g sample of the minced vegetables or fruits is placed in a blender cup, 100 mL of acetone are added and the mixture is shaken vigorously on a mechanical shaker for 30 min. The homogenate is filtered under vacuum through a funnel fitted with a filter paper, and the residue is shaken with 100 mL of acetone and then filtered again. The filtrates are combined and concentrated to about 20 mL using a vacuum rotary evaporator.

(2) Brown rice, wheat and bean

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The cereal samples are milled with an ultracentrifuge mill and sieved through a 42-mesh screen, then 10 g of the sieved sample are transferred into a 300-mL Erlenmeyer flask and soaked in 30 mL of distilled water for 60 min. After 100 mL of acetone have been added, the procedure described for vegetables and fruits is followed.

Residual pendimethalin in various crops was determined as follows.[4] A 10–20-g amount of fruits or vegetables was extracted by blending twice with 200 mL of methanol. Grasses and mint were extracted with 200 mL of methanol–water (1 : 1, v/v). Nuts were extracted with 200 mL of n-hexane–2-propanol (3 : 1, v/v). For the residue analysis of the dinitroaniline herbicides butralin, dinitramine, ethalfluralin, pendimethalin, and trifluralin, a tomato sample (5 g) was extracted twice with 20 mL of methanol in a Sorvall homogenizer and filtered through filter paper.[5] Benfluralin and trifluralin residues in the sample (10 g) were extracted with 100 mL of acetonitrile–water (99 : 1, v/v) in 250-mL screw-cap jars with Teflon liners rotated for 1 h on an end-over-end shaker (40 rpm).[6]

2.2.2 Cleanup

(1) Liquid–liquid partition

(a) NaCl solution–n-hexane partitioning

A 100-mL volume of 5% NaCl aqueous solution and 100 mL of n-hexane are added to the concentrated extracts prepared in Section 2.2.1, and the mixture is shaken vigorously for 5 min. The organic layer is collected, 50 mL of n-hexane are added to

the aqueous layer and the mixture is shaken again. The n-hexane layers are collected, dried with ca 20 g of anhydrous sodium sulfate, concentrated using a vacuum rotary evaporator below 40 °C and dried under a gentle stream of nitrogen.

(b) Acetonitrile–n-hexane partitioning

In the case of plant samples having high oil contents (for example, rice grain, bean, and corn), acetonitrile–n-hexane partitioning is used to remove the oily materials. The concentrated residue obtained in Section (a) above is dissolved in 30 mL of n-hexane and transferred into a separatory funnel, containing 30 mL of acetonitrile, and the mixture is shaken vigorously. The acetonitrile layer is collected and another 30 mL of acetonitrile are added and shaken with the n-hexane layer. The combined acetonitrile phase is carefully evaporated to dryness.

(2) Column chromatography

(a) Solid-phase extraction (SPE) Florisil cartridge cleanup

An SPE Florisil cartridge is pre-washed with 5 mL of n-hexane–diethyl ether (49 : 1, v/v) to remove any contaminants. To separate trifluralin, the concentrated residue obtained in Section (1) (b) above in the flask is loaded in three portions of 5 mL of the same solution on an SPE Florisil cartridge to the eluate containing trifluralin. Pendimethalin is eluted with 30 mL of n-hexane–diethyl ether (19 : 1, v/v) after pre-washing the SPE Florisil cartridge with 10 mL of n-hexane and discarding the eluate from the cartridge three times with 5 mL of n-hexane. The eluate from the SPE Florisil cartridge is evaporated to near dryness below 40 °C and the residue is made up to the appropriate volume with acetone for gas chromatography (GC) analysis.[7]

(b) SPE silica gel cartridge cleanup

Instead of an SPE Florisil cartridge, an SPE silica gel cartridge is also used. After pre-washing the SPE silica gel cartridge with 5 mL of n-hexane, the concentrated residue in the flask is dissolved in 8 mL of n-hexane, the solution is applied to the cartridge, and then the eluate with n-hexane is discarded. Pendimethalin is eluted with 8 mL of n-hexane–diethyl ether (7 : 3, v/v).

(3) Gel permeation chromatography (GPC)

Cleanup of high oil content samples such as nuts, bean, corn, and rice grain was accomplished with GPC, before applying column chromatography. Gelsomino *et al.*[8] analyzed the residues of 48 pesticides including dinitroaniline herbicides with GPC followed by GC. The residues extracted with an organic solvent were dissolved in 3 mL of GPC mobile phase (ethyl acetate–cyclohexane, 1 : 1, v/v) and injected into the GPC column. The purified organic fraction was collected, the collection volume of which was determined from the calibration procedure with corn oil content according to US Environmental Protection Agency (EPA) method No. 3640. For the determination of pendimethalin, the residual sample was transferred to the GPC column, and the pendimethalin-containing eluate of 76–150 mL of cyclohexane–dichloromethane (17 : 3, v/v) was collected and then evaporated to dryness for the next cleanup procedure using column chromatography.[4]

2.2.3 Determination

(1) GC

To determine the residue levels of dinitroaniline herbicides, GC/NPD or GC/ECD is used in general. An aliquot of GC-ready sample solution is injected into the gas chromatograph under the conditions outlined below. Further confirmatory analysis is carried out using gas chromatography/mass spectrometry (GC/MS) in the selected-ion monitoring (SIM) mode.

(a) GC/NPD

Conditions: apparatus, Hewlett-Packard HP5890; column, DB-5 (30 m × 0.53-mm i.d.) with 1.5-μm film thickness; column temperature, 140 °C (1 min), increased at 10 °C min^{-1} to 210 °C; inlet and detector temperature, 250 and 270 °C, respectively; gas flow rates, He carrier gas 20 mL min^{-1}, H_2 3.5 mL min^{-1}, air 130 mL min^{-1}; injection method, splitless mode; injection volume, 2 μL. The retention time of trifluralin is 5.5 min.[7]

Fewer interfering peaks are observed in GC/NPD than in GC/ECD.

(b) GC/ECD

Conditions: apparatus, Hewlett-Packard HP5890; column, DB-17HT (25 m × 0.25-mm i.d.) with 0.15-μm film thickness; column temperature, 100 °C (1 min), increased at 30 °C min^{-1} to 180 °C and then 5 °C min^{-1} to 210 °C; inlet and detector temperature, 250 and 300 °C, respectively; gas flow rates, He carrier gas 1.5 mL min^{-1}, nitrogen make-up gas 60 mL min^{-1}; injection method, splitless mode; injection volume, 2 μL. The retention time of pendimethalin is 8 min.[7]

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(c) Gas chromatography/ion trap detection (GC/ITD)[5,9]

Conditions: apparatus, Perkin-Elmer Model 8500; column, BP-1 (12 m × 0.22-mm i.d.) with 0.25-μm film thickness; column temperature, 85 °C (1 min), increased at 20 °C min^{-1} to 180 °C (1 min) and then at 10 °C min^{-1} to 250 °C; inlet and detector temperature, 270 and 300 °C, respectively; gas flow rate, He carrier gas 10 psig (1 psig = 6895 Pa); injection method, splitless mode; injection volume, 2-μL; mass range, m/z 120–400; scan rate, 0.5 s per scan, 3-μscans; radiofrequency voltage, 1.1 MHz and 0–7.5 kV; automatic gain control, 78 μs–25 ms; solvent delay, 5 min. The retention times of ethalfluralin, trifluralin, dinitramine, butralin and pendimethalin are 6.6, 7.1, 8.1, 10.0 and 10.2 min, respectively.

(d) GC/MS[8]

Conditions: apparatus, Hewlett-Packard HP5890 equipped with an HP5972 mass-selective ion detector (quadruple); column, PTE-5 (30 m × 0.25-mm i.d.) with 0.25-μm film thickness; column temperature, 50 °C (1 min), increased at 20 °C min^{-1} to 150 °C (5 min) and then at 4 °C min^{-1} to 280 °C (30 min); inlet and detector (GC/MS transfer line) temperature, 250 and 280 °C, respectively; gas flow rate, He carrier gas 1 mL min^{-1}; injection method, splitless mode; solvent delay, 3 min; electron ionization voltage, 70 eV; scan rate, 1.5 scans s^{-1}; scanned-mass range, m/z 50–550. The retention times of benfluralin, pendimethalin and trifluralin are 15.2, 25.1 and 15.1 min, respectively. The main ions of the benfluralin mass spectrum were at m/z

292, 264 and 335. Pendimethalin showed the most abundant ion at m/z 252. Trifluralin presented a fragmentation pattern with main ions at m/z 306 and 263.

(2) High-performance liquid chromatography (HPLC)

HPLC has also been used to determine the residue levels of dinitroaniline herbicides.[5,10] Pendimethalin was quantified by HPLC under the following conditions: apparatus, Spectroflow 400 solvent delivery system, Model 430 gradient former, and Kratos Model 783 with UV absorbance detection at 239 nm; column, C_{18} reversed-phase (25 cm × 3.0-mm i.d.); temperature, 40 °C; mobile phase, acetonitrile–water (7 : 3, v/v); flow rate, 1 mL min^{-1}.

2.2.4 Evaluation

Quantitation is performed by the calibration technique. A new calibration curve is constructed with each dinitroaniline standard solution. The peak area is plotted against the injected amount of standard. Each dinitroaniline in the sample is measured by using the peak area for each standard. Before each set of measurements, the sensitivity and stability of the GC and HPLC system is ascertained by injecting more than one standard solution containing ca 0.05–2 mg L^{-1} of each compound.

2.2.5 Recoveries, limit of detection (LOD) and limit of quantitation (LOQ)

The minimum detectable level is estimated with the dinitroaniline signal-to-noise ratios (S/N). With fortification levels between 0.2 and 0.5 mg kg^{-1}, the recovery of trifluralin from plant matrices is 70–99% with the LOD/LOQ being 0.005 mg kg^{-1} according to the analytical method of the Ministry of the Environment, Japan. In multiresidue analysis by GC/NPD, the percent recoveries of pendimethalin from each crop with a fortification level of 0.25 mg kg^{-1} were brown rice 70, potato 70, cabbage 80, lettuce 89, carrot 84, cucumber 64, shiitake 74, apple 76, strawberry 99, and banana 99%. The LOD for each sample was 0.01 mg kg^{-1} for pendimethalin.[11]

In residue analysis by GC/ECD, recoveries of the majority of dinitroaniline herbicides from fortified samples of carrot, melon, and tomato at fortification levels of 0.04–0.10 mg kg^{-1} ranged from 79 to 92%. The LODs were benfluralin 0.001, pendimethalin 0.002 and trifluralin 0.001 mg kg^{-1} for the GC/ECD method.[8]

The recoveries of five herbicides (ethalfluralin, trifluralin, dinitramine, butralin, and pendimethalin) added to tomato in the range 0.1 to 1 mg kg^{-1} were determined using GC-ITD. The average recoveries ranged from 84 to 104%, and the detection limit of these compounds was near 0.01 mg kg^{-1}.[5]

GC/MS in the SIM mode was carried out for confirmation of all positive and ambiguous results obtained from GC analysis. GC/MS was effective as a multiresidue screening method for crops; the mean recovery of trifluralin from green bean, cilantro, apple, tomato, and green onion at a fortification level of 0.25 mg kg^{-1} was 55% and the LOD was 0.05 mg kg^{-1}.[12]

With the HPLC method, the recovery of pendimethalin from turf grass at a fortification level of 0.25 mg kg^{-1} was 97% and the LOD was 0.001 mg kg^{-1}.[10]

2.2.6 Calculation of residues

The amount of dinitroaniline herbicide residue (R, mg kg^{-1}) in the sample is calculated with the following equation:

$$R = (W_i / V_i)/(V_f / G)$$

where

G = sample weight (g)
V_i = injection volume into the gas chromatograph (μL) or high-performance liquid chromatograph (μL)
V_f = final sample volume (mL)
W_i = amount of dinitroaniline herbicide for V_i read from the calibration curve (ng)

3 Analytical methodology for soil

3.1 Nature of the residues

Dinitroaniline herbicides are generally stable in soil. Residue methods were developed for both the parent molecule and selective soil degradates.

3.2 Method principle

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Air-dried soil samples were screened through a 2-mm sieve, then the water content in the soil was calculated after holding the soil samples for 5 h at 105 °C.

Residual dinitroaniline herbicides are generally extracted from 10–25 g of air-dried soil samples using organic solvents such as ethyl acetate, acetonitrile, methylene chloride and acetone by sonication, mechanical shaking or Soxhlet extraction. If necessary, the extract is then cleaned by a Florisil column or SPE. The extract is allowed to evaporate completely to dryness and the residue is dissolved in an appropriate volume of the solvent for GC or HPLC analysis.

3.2.1 Extraction and cleanup

A 20-g sample of air-dried soil is extracted with 100 mL of ethyl acetate in a flask shaker for 45 min. After shaking, the extract is decanted and separated. The soil is re-extracted with 100 mL of ethyl acetate for 45 min. The combined soil extracts are filtered through a Whatman No 1 filter paper and the filter cake is washed with an additional 20 mL of ethyl acetate. The extracts are evaporated nearly to dryness, under vacuum, using a rotary evaporator. The residue is dissolved in an appropriate volume before GC analysis.[5]

Garimella *et al.*[13], investigated the effect on trifluralin recovery of different extraction methods. A supercritical fluid extraction (SFE) procedure for the isolation of the analytes from the matrices with a commercial SFE system (Dionex Model 703)

was developed, and for analyte collection, C_{18} traps (Dionex) were used. The collection tube was activated by passing methanol (1 mL) and acetone (1 mL), successively. The extraction of 3 g of soil sample was conducted at 20.3 mPa for 3 min and then at 34.4 mPa for 17 min using highly purified CO_2. The oven temperature was maintained at 60 °C and the restrictor temperature at 125 °C. As a co-solvent, 15% (v/v) acetone in CO_2 was used and the analyte was collected and transferred on to the SPE tube. The SPE extract was used for GC or HPLC analysis. In the case of liquid vortex extraction, 10 g of soil sample were vortexed three times for 2 min with 20 mL of acetone and equilibrated overnight. The samples were then vortexed four times for 10 s and centrifuged at 870 *g*, and the supernatant was collected for GC or HPLC analysis. Soxtec extraction was performed on an automated Soxhlet (Tecator HT 1045 and HT2 1046 Soxtec). Samples of 5 g of soil in the extraction thimbles were placed in the Soxtec apparatus with 75 mL of acetone in the extraction cup. The temperature was set at 130 °C and the samples were boiled for 20 min followed by 15 min of rinsing. The acetone extract was concentrated for GC or HPLC analysis. By comparing the extraction efficiencies with these extraction methods, SFE and liquid vortex extraction of trifluralin from soil samples were determined to be preferable to Soxtec extraction.

SPE purification was carried out continually after the SFE procedure. The SPE tube was mounted on a vacuum manifold and preconditioned with 2 mL acetone and 2 mL of acetone–water (2 : 1, v/v) successively. The tube was connected with a 25-mL reservoir into which the extract was transferred. After percolation, the tube was rinsed with 10 mL of water–acetone (2 : 1, v/v) and the sorbent was dried under vacuum for 15 min. The residue was eluted with 5 mL of acetone into a volumetric flask. As well as water–acetone (2 : 1, v/v), 2 mL of acetonitrile–water (1 : 1, v/v) were also used for rinsing.[6,13]

3.2.2 Determination and evaluation

The determination of the residue levels by GC and HPLC and evaluation of the residue levels were carried out by the procedures described for the plant material in Sections 2.2.3 and 2.2.4, respectively.

3.2.3 Recoveries, limit of detection and limit of quantitation

For the determination of five herbicides (ethalfluralin, trifluralin, dinitramine, butralin, and pendimethalin) at fortification levels between 0.1 and 1 mg kg^{-1}, soil was extracted with ethyl acetate and the extract was purified on a Florisil column. The residues were eluted with acetone and then analyzed by GC. The average recoveries varied from 75 to 111% for GC/NPD and from 88 to 98% for GC/ITD with the LOD being 0.01 mg kg^{-1} for both GC/NPD and GC/ITD.[5] The recoveries of pendimethalin at fortification levels ranging from 0.2 to 1 mg kg^{-1} determined by GC/NPD were between 96 and 101% and the LOD was lower than 0.01 mg kg^{-1}.[9]

In the HPLC method for the simultaneous determination of dinitramine, ethalfluralin, trifluralin, pendimethalin, and isopropalin, a Spherisorb ODS-2 column (25 cm × 4.6-mm i.d.) was used; the mobile phase was acetonitrile–water (11 : 9, v/v) at a flow rate of 1.0 mL min^{-1} with UV absorbance detection at 220 nm. The average

recoveries for extraction with diethyl ether from soil were in the range 89–104% and the LOD for these herbicides was 0.02 mg kg^{-1}.[14]

3.2.4 Calculation of residues

Calculation of residues in soil was carried out as described in Section 2.2.6.

3.3 Analytical method for soil metabolites

The fate of the dinitroaniline herbicides in soil is extremely complex and many metabolites have been identified. Golab and Althaus[15] reported 28 metabolites identified in a degradation study of trifluralin in soil. Major degradation products of dinitroaniline herbicides were formed by nitro reduction, *N*-dealkylation (monodealkylated and completely dealkylated) and the ring formation of benzimidazole.

Analytical methods for fortified soils were developed for the simultaneous quantitation of the trifluralin metabolites, 2,6-dinitro-*N*-propyl-4-(trifluoromethyl)-benzenamine (**1**) and 2,4-dinitro-*N*,*N*-dipropyl-6-(trifluoromethyl)benzenamine (**2**)[13] (Figure 2). The SFE method developed as described in Section 2.2.1 was extended to the determination of these metabolites. From soil fortified with 0.5–2.5 mg kg^{-1} each of trifluralin, (**1**) and (**2**), the compounds were efficiently extracted by this procedure. Trifluralin and its metabolites (**1**) and (**2**) are characterized by absorbance bands in both the ultraviolet (UV) and visible ranges for HPLC; however,

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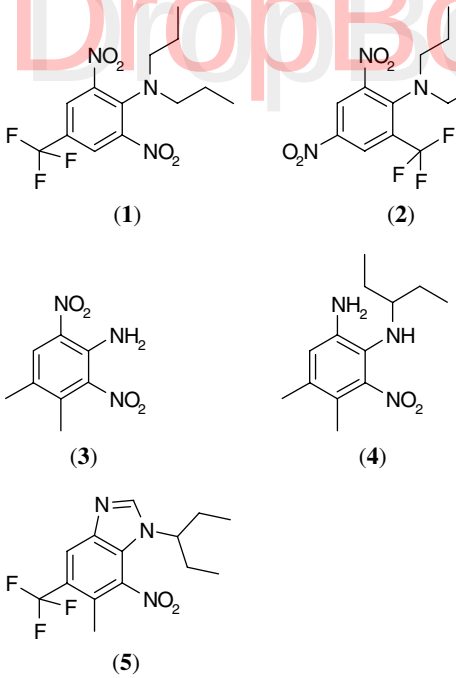


Figure 2 Structures of metabolites of trifluralin (**1** and **2**) and pendimethalin (**3**–**5**)

in the research by Garimella *et al.*,[13] these compounds were monitored simultaneously in the visible range at 386 nm. This wavelength permitted the use of acetone, which had a UV cutoff at 330 nm, as the mobile phase [acetone–water (4 : 1, v/v)].

The predicted degradation products of pendimethalin, via dealkylated pendimethalin (3,4-dimethyl-2,6-dinitroaniline) (**3**), partially reduced pendimethalin [*N*-(1-ethylpropyl)-3,4-dimethyl-2-nitro-1,6-diaminobenzene] (**4**) and cyclized product [*N*-(1-ethylpropyl)-5,6-dimethyl-7-nitrobenzimidazole] (**5**) (Figure 2) were determined. A 100-g sample of soil was extracted with 300 mL of methanol–concentrated HCl (49 : 1, v/v) by shaking for 1 h. The extract was concentrated and partitioned with n-hexane for pendimethalin and with n-hexane–ethyl acetate (1 : 1, v/v) for pendimethalin degradation products and these compounds were detected by GC/ECD. The GC conditions were as follows: column, megabore column packed with 3% OV-17; inlet, column and detector temperatures, 235, 210 and 275 °C, respectively; gas flow rate, nitrogen 20 mL min^{-1}. With an injection volume of 3 μL, the retention times were 3.5 min for pendimethalin, 1.8 min for (**3**), 3.0 min for (**4**) and 3.6 min for (**5**). The recoveries of pendimethalin and degradation products with fortification levels ranging from 0.2 to 1 mg kg^{-1} determined by GC/ECD were more than 85% for soil. A linear response was obtained between 0.1 and 5 ng.[16]

4 Analytical methodology for water

4.1 Nature of the residues

Dinitroaniline herbicides have low soil mobility potential. Herbicide residues in the treated field are usually incorporated into the upper layers of the soil mainly as unextractable bound residue; therefore, the movement of dinitroaniline herbicides from soil to the water compartment is minimal. Run-off is the principal route, which could lead to the contamination of surface waters. Residue methods were developed to measure the parent concentration in water samples.

4.2 Analytical method

Water (1000 mL) is transferred into a 2-L separatory funnel and extracted with two portions of 50 mL of dichloromethane for 30 min with a mechanical shaker, and the extracts are collected in a 200-mL Erlenmeyer flask. The combined extracts are filtered through anhydrous sodium sulfate into a 300-mL round-bottom flask and evaporated to dryness with a rotary evaporator under vacuum. The residue is dissolved in 1 mL of n-hexane and an aliquot is analyzed by GC/NPD or GC/ITD under the conditions described in Section 2.2.3.[9] Recoveries from water samples fortified with 0.0002 and 0.001 mg L^{-1} of pendimethalin were in the range 94–110% by GC/NPD and 91–111% by GC/ITD. The detection limit was lower than 0.0001 mg L^{-1} with both methods.

Cabras *et al.*[14] reported an HPLC residue method for dinitroaniline herbicides. A water sample was analyzed after purification and concentration on a Bond-Elut C_{18} cartridge (500-mg/2.8-mL). The cartridge was treated with 10 mL of methanol

followed by 10 mL of water. A 100-mL water sample was added to the cartridge using a reservoir and the cartridge was allowed to percolate slowly (1 mL min^{-1}). The reservoir was removed and the cartridge was washed with 5 mL of methanol–water (1 : 1, v/v), followed by 5 mL of water. The cartridge was air-dried under vacuum for 2 min and then the dinitroaniline herbicides were eluted with 2 mL of diethyl ether. The extract was evaporated completely to dryness and the residue was dissolved in 1 mL of mobile phase, acetonitrile–water (3 : 1, v/v). The recoveries from water samples fortified with 0.002 mg L^{-1} of dinitramine, ethalfluralin, isopropalin, pendimethalin, and trifluralin were 89–104%. The LOD was 0.0005 mg L^{-1} in water for the five herbicides.

Calculation of residues in water was carried out as described in Section 2.2.6 for plant material.

References

1. T.R. Roberts, D.H. Hutson, P.W. Lee, P.H. Nicholls, and J.R. Plimmer, 'Metabolic Pathways of Agrochemicals,' Royal Society of Chemistry, Cambridge (1998).
2. Ministry of Health, Labor and Welfare, Japan, 'Specifications and Standards for Food, etc.,' Ministry of Health, Labor and Welfare, Japan, Tokyo.
3. Ministry of the Environment, 'Analytical Method for Trifluraline, Notification of Ministry of the Environment, Japan,' Ministry of the Environment, Tokyo (1976) (in Japanese).
4. J. Engebretson, G. Hall, M. Hengel, and T. Shibamoto, *J. Agric. Food Chem.,* **49**, 2198 (2001).
5. A.I. Garcia-Valcarcel, C. Sanchez-Brunete, L. Martinez, and J.I. Tadeo, *J. Chromatogr.,* **719**, 113 (1996).
6. A.A. Krause and H.D. Niemczyk, *J. Environ. Sci. Health,* **B27**, 39 (1992).
7. M. Ueji, H. Kobayashi, and K. Nakamura, 'Analytical Methods for Pesticide Residues,' Soft Science, Tokyo, pp. 397, 508 (2001) (in Japanese).
8. A. Gelsomino, B. Petrovicova, S. Tiburtini, E. Magnaniand, and M. Felici, *J. Chromatogr.,* **782**, 105 (1997).
9. C. Sanchez-Brunete, L. Martine, and J.L. Tadeo, *J. Agric. Food Chem.,* **42**, 2210 (1994).
10. J.J. Gasper, J.R. Street, S.K. Harrison, and W.E. Pound, *Weed Sci.,* **42**, 586 (1994).
11. Y. Nakamura, Y. Tonogai, Y. Sekiguchi, Y. Tsumura, N. Nishida, K. Takakura, M. Isechi, K. Yuasa, M. Nakamura, N. Kifune, K. Yamoto, S. Terasawa, T. Oshima, M. Miyata, K. Kamakura, and Y. Ito, *J. Agric. Food Chem.,* **42**, 2508 (1994).
12. W. Liao, T. Joe, and W.G. Cusick, *J. Assoc. Off. Anal. Chem.,* **74**, 554 (1991).
13. U.I. Garimella, G.K. Stearman, and M.J.M. Wells, *J. Agric. Food Chem.,* **48**, 5874 (2000).
14. P. Cabras, M. Melis, L. Spanedda, and C. Tuberoso, *J. Chromatogr.,* **585**, 164 (1991).
15. T. Golab and W.A. Althaus, *J. Agric. Food Chem.,* **27**, 163 (1979).
16. G. Kulshrestha, S.B. Singh, S.P. Lal, and N.T. Yaduraju, *Pestic. Manage. Sci.,* **56**, 202 (2000).

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Sulfonylurea herbicides

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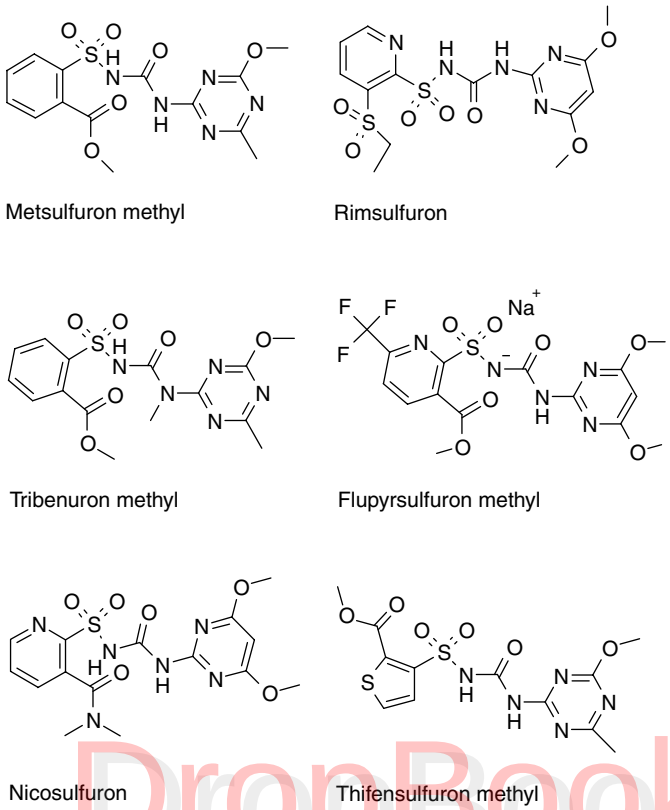
1 Introduction

Sulfonylurea herbicides were first introduced in 1982 by DuPont Crop Protection. They are typically applied at rates less than 100 g ha^{-1}, have low mammalian toxicity, and degrade to innocuous compounds after application. They are used to control a variety of broad-leafed weeds and grasses in cereals and other row crops and for industrial weed control. The biological mode of action is via inhibition of acetolactate synthase (ALS), an enzyme that is found in plants, but not in animals.[1] Approximately 25 sulfonylurea herbicides are currently registered for agricultural uses on a global basis. A few examples of these molecules are shown in Figure 1. The compounds are characterized by the presence of a sulfonylurea bridge between two heterocyclic moieties. Sulfonylureas are both chemically and thermally unstable. Rapid hydrolytic cleavage of the sulfonylurea bridge occurs in aqueous acidic solutions; most sulfonylureas demonstrate improved stability and solubility in aqueous neutral to slightly alkaline solutions, where they exist in the anionic form through the loss of one of the urea hydrogen atoms. These compounds, generally, are stable in organic solvents and are soluble at levels greater than 1 mg per 100 mL in most common organic solvents, with the exception of hydrocarbons.

Sulfonylureas are not directly amenable to gas chromatography (GC) because of their extremely low volatility and thermal instability. GC has been used in conjunction with diazomethane derivatization,[2,3] pentafluorobenzyl bromide derivatization,[4] and hydrolysis followed by analysis of the aryl sulfonamides.[5] These approaches have not become widely accepted, owing to poor performance for the entire family of sulfonylureas. Capillary electrophoresis (CE) has been evaluated for water analysis[6–8] and soil analysis.[9] The low injection volumes required in CE may not yield the required sensitivity for certain applications. Enzyme immunoassay has been reported for chlorsulfuron[10] and triasulfuron,[11] with a limit of detection (LOD) ranging from 20 to 100 ng kg^{-1} (ppt) in soil and water.

The most common approaches to sulfonylurea determinations involve high-performance liquid chromatography (HPLC). The earliest reported methods utilized normal-phase liquid chromatography (LC) with photoconductivity detection;[12,13] this type of detector demonstrated undesirably long equilibration times and is no longer

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Figure 1 Structures of selected sulfonylurea herbicides

commercially available. More recent methods use reversed-phase HPLC with either ultraviolet (UV) or mass spectrometry (MS) detection. HPLC/UV methods have been reported for the determination of selected sulfonylureas in soil and water;[14–16] in many cases, the sensitivity of these methods is not adequate, and the lack of specificity usually requires extensive cleanup and/or complicated column-switching arrangements and mobile phase gradients. Early applications of HPLC with MS detection [liquid chromatography/mass spectrometry (LC/MS)] involved thermospray ionization,[17] fast-atom bombardment,[18,19] and direct liquid introduction.[20] However, quantitative determination of sulfonylureas by LC/MS was not widespread until electrospray interfaces were developed.[21–24] The current best practice for trace-level sulfonylurea determination in biological and environmental matrices is HPLC with a positive-ion electrospray interface and tandem mass spectrometry (MS/MS) detection of at least one parent to daughter ion transition using either an ion trap or, preferably, a triple-quadrupole mass spectrometer. The sensitivity and selectivity obtained by liquid chromatography/tandem mass spectrometry (LC/MS/MS) meets the most stringent regulatory criteria for detection, quantitation, and confirmation.[25,26] All of the procedures summarized in this article recommend LC/MS/MS as the means of detection for these reasons.

2 Analytical methodology

LC/MS/MS is the preferred means of detection, quantitation, and confirmation of sulfonylurea herbicides in biological and environmental matrices. Therefore, recommendations for establishing and optimizing LC/MS/MS analyses common to all matrices are given first, followed by specific rationales for methods and sample preparation techniques for plant, soil, and water matrices.

2.1 LC/MS/MS analysis

A triple-quadrupole mass spectrometer with an electrospray interface is recommended for achieving the best sensitivity and selectivity in the quantitative determination of sulfonylurea herbicides. Ion trap mass spectrometers may also be used, but reduced sensitivity may be observed, in addition to more severe matrix suppression due to the increased need for sample concentration or to the space charge effect. Also, we have observed that two parent to daughter transitions cannot be obtained for some of the sulfonylurea compounds when ion traps are used in the MS/MS mode. Most electrospray LC/MS and LC/MS/MS analyses of sulfonylureas have been done in the positive ion mode with acidic HPLC mobile phases. The formation of $(M + H)^+$ ions in solution and in the gas phase under these conditions is favorable, and fragmentation or formation of undesirable adducts can easily be minimized. Owing to the acid–base nature of these molecules, negative ionization can also be used, with the formation of $(M - H)^-$ ions at mobile phase pH values of approximately 5–7, but the sensitivity is often reduced as compared with the positive ion mode.

Reversed-phase liquid chromatography with a C_8, phenyl, or C_{18} column (or equivalent) is recommended. A binary pumping system capable of producing a linear gradient is sufficient. Water and methanol, both acidified with equal amounts of acetic acid, are used to form the gradient. Table 1 provides an example of HPLC conditions used for the determination of 13 sulfonylureas with a wide range of polarity. In this example, aqueous samples (100 μL) are injected at a weak mobile phase composition to facilitate on-column focusing, followed by a steep gradient to facilitate removal of as many of the matrix components as possible. The analytes are then eluted between 11 and 16 min, followed by cleaning and re-equilibration periods. Baseline resolution of the analytes is not obtained and is not necessary since they all have different mass spectral transitions. A switching valve is used to divert the HPLC effluent to waste before and after the 11–16-min time period, in order to reduce source contamination and to enable more samples to be analyzed before the source needs to be cleaned. Since the electrospray interface works optimally at low flow rates, the HPLC flow is split post-column such that only 100 μL min^{-1} actually passes through the interface (approximately 10 : 1 split), while the remainder is diverted to a waste container.

The MS/MS response for each analyte must first be optimized on the specific instrument to be used. This is usually done by infusing a solution of the analyte into the HPLC mobile phase without a column present. The composition of the mobile phase should match that expected at the time of analyte elution within ±25%. The instrument is first operated in the LC/MS mode, and the settings for the electrospray interface are

Table 1 Example HPLC conditions for the determination of sulfonylurea herbicides by LC/MS/MS

System	Agilent 1100 HPLC		
Column	4.6-mm i.d. ×15 cm, Phenomenex C_8 analytical column with 3-μm-diameter packing		
Column temperature	40 °C		
Injection volume	0.100 mL		
Autosampler temperature	4 °C		
Flow rate	1.0 mL min^{-1}		
Conditions	Time	A[a] (%)	B[a] (%)
	0.0	75	25
	2.0	75	25
	12.0	30	70
	15.0	20	80
	16.0	10	90
	18.0	10	90
	18.5	75	25
	23.0	75	25
Analyte	Retention time (min)		
Nicosulfuron	12.0		
Sulfometuron methyl	12.3		
Thifensulfuron methyl	12.4		
Metsulfuron methyl	12.9		
Ethametsulfuron methyl	13.0		
Rimsulfuron	13.2		
Tribenuron methyl	13.7		
Chlorsulfuron	14.2		
Bensulfuron methyl	14.6		
Azimsulfuron	14.6		
Triflusulfuron methyl	15.6		
Chlorimuron ethyl	15.8		
Flupyrsulfuron methyl	15.9		
Total run time	23.0		

[a]A = 0.05% acetic acid; B = 0.05% acetic acid in methanol.

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optimized to provide maximum response for the $(M + H)^+$ ion; this process is usually automated. Then, the settings for the collision cell are optimized to produce maximum response of one or two characteristic daughter ions. Most modern instruments allow this to be done automatically. In general, sulfonylureas are very amenable to positive ion electrospray MS and MS/MS analysis, with excellent sensitivity compared with most other agrochemicals, and optimal responses can be easily obtained by proficient operators.

A minimum number of transitions should be monitored at any given time during the course of analysis. If only a few well-resolved peaks are to be monitored, the groups of ions to be acquired may easily be changed so optimum sensitivity is obtained. If many closely eluting or even overlapping chromatographic peaks are to be monitored, acquiring too many signals at any given time will result in more poorly

Table 2 Suggested ion transitions for the determination of sulfonylurea herbicides by LC/MS/MS

Analyte	Primary (quantitative) transition	Secondary (confirmatory) transition
Nicosulfuron	$410.9 \rightarrow 213.0 \pm 0.2$	$410.9 \rightarrow 182.0 \pm 0.2$
Sulfometuron methyl	$365.0 \rightarrow 150.0 \pm 0.2$	$365.0 \rightarrow 199.0 \pm 0.2$
Thifensulfuron methyl	$387.9 \rightarrow 167.0 \pm 0.2$	$387.9 \rightarrow 205.0 \pm 0.2$
Metsulfuron methyl	$382.0 \rightarrow 167.0 \pm 0.2$	$382.0 \rightarrow 199.0 \pm 0.2$
Ethametsulfuron methyl	$410.9 \rightarrow 196.0 \pm 0.2$	$410.9 \rightarrow 168.0 \pm 0.2$
Rimsulfuron	$431.9 \rightarrow 182.0 \pm 0.2$	$431.9 \rightarrow 325.0 \pm 0.2$
Chlorsulfuron	$358.0 \rightarrow 167.0 \pm 0.2$	$358.0 \rightarrow 141.0 \pm 0.2$
Azimsulfuron	$425.0 \rightarrow 182.0 \pm 0.2$	$425.0 \rightarrow 244.0 \pm 0.2$
Bensulfuron methyl	$411.0 \rightarrow 149.0 \pm 0.2$	$411.0 \rightarrow 182.0 \pm 0.2$
Flupyrsulfuron methyl	$465.9 \rightarrow 182.0 \pm 0.2$	$465.9 \rightarrow 139.0 \pm 0.2$
Chlorimuron ethyl	$415.0 \rightarrow 186.0 \pm 0.2$	$415.0 \rightarrow 83.0 \pm 0.2$
Triflusulfuron methyl	$493.0 \rightarrow 264.0 \pm 0.2$	$493.0 \rightarrow 96.0 \pm 0.2$
Triasulfuron	$401.8 \rightarrow 167.1 \pm 0.2$	$401.8 \rightarrow 141.1 \pm 0.2$
Cinosulfuron	$413.9 \rightarrow 183.1 \pm 0.2$	$413.9 \rightarrow 215.1 \pm 0.2$
Amidosulfuron	$370.1 \rightarrow 261.0 \pm 0.2$	$370.1 \rightarrow 218.0 \pm 0.2$
Oxasulfuron	$407.0 \rightarrow 150.0 \pm 0.2$	$407.0 \rightarrow 210.1 \pm 0.2$
Sulfosulfuron	$471.1 \rightarrow 210.9 \pm 0.2$	$471.1 \rightarrow 260.8 \pm 0.2$
Prosulfuron	$419.9 \rightarrow 141.1 \pm 0.2$	$419.9 \rightarrow 167.1 \pm 0.2$
Halosulfuron methyl	$430.8 \rightarrow 182.1 \pm 0.2$	$430.8 \rightarrow 222.1 \pm 0.2$
Primisulfuron methyl	$468.8 \rightarrow 254.1 \pm 0.2$	$468.8 \rightarrow 199.1 \pm 0.2$

defined peaks with noticeable decreases in resolution. If this is observed, adjustment of chromatographic conditions to improve the resolution of some of the analytes is recommended, so fewer signals have to be acquired at once. This is especially true if two parent-to-daughter ion transitions per analyte are to be acquired.

If sulfonylurea herbicides can reasonably be expected to be present in an analytical sample (based on prior knowledge), one parent-to-daughter ion transition is usually considered sufficient to confirm its presence. In other cases where little is known about the sample history, two parent-to-daughter ion transitions are generally considered to be necessary for a definitive confirmation. Suggested ion transitions for most of the registered sulfonylurea herbicides are listed in Table 2. Furthermore, the ratio of the signals for the two transitions obtained for the sample should match that of an authentic standard within $\pm 30\%$, at most.[25,26]

At least four chromatographic standards prepared at concentrations equivalent to 50–70% of the limit of quantitation (LOQ) up to the maximum levels of analytes expected in the samples should be prepared and analyzed concurrently with the samples. In LC/MS/MS analysis, the first injection should be that of a standard or reagent blank and should be discarded. Then, the lowest standard should be injected, followed by two to four blanks, control samples, fortifications or investigation samples, followed by another chromatographic standard. This sequence is then repeated until all the samples have been injected. The last injection should be that of a standard. In order to permit unattended analysis of a normal analysis set, we recommend that samples and standards be made up in aqueous solutions of ammonium acetate (ca 5 mM) with up to 25% of an organic modifier such as acetonitrile or methanol if needed. In addition, use of a chilled autosampler maintained at 4 °C provides additional prevention of degradation during analysis.

2.2 Crops, food and feed

2.2.1 Nature of the residue

Sulfonylurea herbicides are generally applied to crops as an early post-emergent herbicide. Crops that are tolerant to these herbicides quickly metabolize them to innocuous compounds. At maturity, residues of the parent compound in food and feed commodities are nondetectable. Metabolites are not considered to be of concern, and their levels are usually nondetectable also. For this reason, the residue definition only includes the parent compound. Tolerances [or maximum residue limits (MRLs)] are based on the LOQ of the method submitted for enforcement purposes and usually range from 0.01 to 0.05 mg kg^{-1} (ppm) for food items and up to 0.1 mg kg^{-1} for feed items. There is no practical need for residue methods for animal tissues or animal-derived products such as milk, meat, and eggs. Sulfonylurea herbicides are not found in animal feed items, as mentioned above. Furthermore, sulfonylurea herbicides intentionally dosed to rats and goats are mostly excreted in the urine and feces, and the traces that are absorbed are rapidly metabolized to nontoxic compounds. For this reason, no descriptions of methods for animal-derived matrices are given here.

2.2.2 Rationale for methods

Sulfonylurea herbicides can be conveniently extracted from watery and dry plant materials such as vegetables and cereal and corn grain, straw and forage using aqueous buffers adjusted to pH 6.0–7.0. In this pH range, the sulfonylureas exist in the predominantly anionic form, where they exhibit maximum stability and solubility in aqueous solutions. At lower pH values, there is an increased tendency for dissociation by hydrolysis. The most acid-sensitive sulfonylurea is tribenuron methyl, which completely hydrolyzes in aqueous acidic solutions within 1 day. At pH > 7.0, a few of the sulfonylureas such as rimsulfuron and flupyrsulfuron methyl undergo an irreversible rearrangement to bridge-condensed products. Also, the possibility of increased co-extractives from the matrix is possible at alkaline pH. Purely aqueous buffers are convenient for extraction purposes, and extraction efficiency studies conducted with aged carbon-14 crop residues indicate that endogenous residues are completely recovered from watery and dry plant material. In the case of dry plant material, the sample is soaked in the aqueous buffer prior to homogenization. Addition of organic co-solvents such as methanol or acetonitrile does not appear to be necessary for the extraction of watery or dry plant samples and usually makes cleanup more difficult owing to increased co-extractives and decreased retention of the analytes during solid-phase extraction (SPE) cleanup using hydrophobic adsorbents.

Aqueous extracts are normally concentrated and purified using hydrophobic SPE sorbents. We have obtained the best results with graphitized carbon sorbents; most plant pigments and starches are strongly retained on these cartridges while the sulfonylurea analytes can be eluted with acidified methanol–dichloromethane. We have observed that at least one sulfonylurea, triflusulfuron methyl, is degraded or irreversibly adsorbed on these cartridges, so alternatives with C_{18} or polystyrene–divinyl benzene cartridges are also available. In these cases, additional cleanup using strong anion-exchange cartridges is often necessary.

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Oily crops such as soybeans and canola (oilseed rape) cannot be extracted with aqueous buffers, because the extraction solvent cannot permeate the hydrophobic plant tissue matrix. In these cases, homogenization in acetonitrile–hexane is recommended. This solvent mixture is able to extract sulfonylureas from these samples with a minimum of co-extracted oil. After extraction, the sulfonylureas partition into the acetonitrile phase while most of the oil stays in the hexane phase. Further cleanup is accomplished using a silica SPE cartridge and normal-phase conditions.

Analysis of the concentrated, purified sample extracts is effected by LC/MS or LC/MS/MS, as described in Section 2.1.

2.2.3 Description of methods

Fortifications are made by pipetting 100–500 μL of the appropriate standards in acetonitrile on to the sample (10 g) before any extraction solution is added and then allowing the sample to air dry for 30 min.

Watery and dry crops. Watery and dry crop samples (10 g) are extracted by homogenization in 2 × 90 mL of 20 mM, pH 6.0 potassium phosphate buffer. Dry crop samples are allowed to soak (refrigerated) for 60 min in the first 90 mL of buffer before homogenization in order to hydrate the matrix. After each homogenization using a Tissumizer (Tekmar, Cincinnati, OH, USA) or similar equipment, the sample is centrifuged, and the supernatants are combined. The final volume of the combined supernatants is adjusted to 200 mL with water. An Envi-Carb cartridge (1-g/12-mL; Supelco, Bellefonte, PA, USA) is preconditioned with 10 mL of 0.1 N formic acid in methanol–dichloromethane (1 : 9, v/v), 10 mL of methanol, 10 mL of 0.1 N HCl, and finally 15 mL of water. The cartridge is not allowed to become dry during or after preconditioning. A 10-mL aliquot of the sample extract is passed through the cartridge, and the charge is discarded. The cartridge is washed with 10 mL of water and 5 mL of methanol; both washes are discarded. Air is allowed to pass through the cartridge briefly (several seconds) after charging and washing and then for an additional 2 min under maximum vacuum after the last wash. The sulfonylureas are eluted with 20 mL of 0.1 N formic acid in methanol–dichloromethane (1 : 9, v/v). The eluate is evaporated to dryness under a gentle stream of dry nitrogen at 35 °C and reconstituted in 2–5 mL of acetonitrile–5 mM ammonium acetate solution (1 : 9, v/v).

If the analytes of interest are not quantitatively recovered from a graphitized carbon SPE cartridge (such is the case for triflusulfuron methyl), an alternative cleanup step using strong anion-exchange (SAX) and polystyrene–divinylbenzene SPE cartridges is available. In the case of triflusulfuron methyl, 2 g of sample (watery or dry crop) are homogenized in 15 mL of acetonitrile–0.1 M ammonium carbonate (2 : 1, v/v). NaCl (2.5 g) is added to induce phase separation, and the sample is again homogenized. The sample is centrifuged, and the acetonitrile and aqueous phases are allowed to separate. The sulfonylureas partition into the acetonitrile layer. An SAX SPE cartridge (500-mg/6-mL, available from various suppliers) is preconditioned with 2.5 mL of methanol and 2.5 mL of acetonitrile–0.1 N acetic acid (3 : 7, v/v). An Oasis HLB cartridge (500-mg/12-mL; Waters, Bedford, MA, USA) is preconditioned with 5 mL of methanol and 5 mL of acetonitrile–0.1 N acetic acid (3 : 7, v/v). The SAX cartridge is attached to the top of the Oasis cartridge. A 25% aliquot of the acetonitrile layer from

the sample extract is diluted threefold with 0.1 N acetic acid and passed through the stacked cartridges. The cartridges are rinsed with 20 mL of acetonitrile–0.1 N acetic acid (3 : 7, v/v). The SAX cartridge is allowed to dry at this point in the procedure and is removed and discarded. The Oasis cartridge is washed with an additional 5 mL of acetonitrile–0.1 N acetic acid (3 : 7, v/v) and is allowed to go to dryness and air dry under vacuum for 10 min. The cartridge is eluted with 12 mL of acetonitrile. The eluate is evaporated to dryness under a stream of nitrogen and reconstituted in 2.5 mL of methanol–5 mM ammonium acetate solution (1 : 3, v/v). The sample is now ready for analysis by LC/MS/MS.

Oily crops. Oily crop samples, such as canola seed and soybean, are extracted by homogenization of 5 g of seed sample in 120 mL of hexane-saturated acetonitrile plus 40 mL of acetonitrile-saturated hexane. The resulting extract is centrifuged to separate the two layers, and a 6-mL aliquot of the acetonitrile layer is evaporated to dryness under a stream of nitrogen at ambient temperature. The sample is reconstituted in 4 mL of ethyl acetate. A silica SPE cartridge (5-g/20-mL, Silica Mega-Bond Elut; Varian Sample Preparation, Harbor City, CA, USA) is preconditioned with 20 mL of ethyl acetate. The 4-mL sample is applied, followed by 2 × 1-mL rinses of the sample tube with ethyl acetate; the charge and wash are discarded. The cartridge is washed with an additional 15 mL of ethyl acetate and 20 mL of ethyl acetate–2-propanol–methanol solvent mixture (15 : 4 : 1, v/v/v), which is discarded. The analytes are eluted with 15 mL of ethyl acetate (containing 0.5% acetic acid), the eluate is evaporated to dryness under a stream of nitrogen at ambient temperature, and the residue is reconstituted in 3 mL of methanol–10 mM ammonium acetate (3 : 17, v/v) for LC/MS/MS analysis. The silica SPE cleanup described above was optimized for ethametsulfuron methyl; the ethyl acetate–2-propanol–methanol wash and the eluting solvent may have to be optimized for sulfonylureas of different polarities.

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2.3 Soil

2.3.1 Nature of the residue

The degradation rate of sulfonylureas in soil is dependent on many factors, including soil properties, temperature, and the chemical stability of the compound itself. In general, sulfonylureas do not have a tendency to accumulate in soil from season to season. Both chemical and microbial degradation can occur, and the degradation products do not show any significant herbicidal activity or have toxicological concerns. For this reason, the intact sulfonylureas are the only compounds that are monitored in normal practice, and the methodology description will exclude metabolites. A recent study was conducted by DuPont that evaluated the sensitivity of various nontarget plant species to a variety of sulfonylurea herbicides.[27] The results of this study indicate that application rates of less than 0.1 g of active ingredient (ai) per hectare (ha) do not measurably affect yield and/or quality of sensitive species; only 20% of the trials produced measurable effects when application rates of 0.1–0.5 g ai ha^{-1} were used. Using the approximation that 0.1 g a.i. ha^{-1} results in soil concentrations of 0.1 $\mu g\,kg^{-1}$ (ppb), an LOQ of 0.05 $\mu g\,kg^{-1}$ would be sufficiently sensitive for the determination of all sulfonylureas in soil.

2.3.2 Rationale for methods

Ammonium carbonate solution containing a small amount (ca 10%) of methanol are optimal for extracting aged residues of sulfonylurea herbicides. Some of the sulfonylureas have a tendency to bind to clay in certain soils, and the counter ions provided by ammonium carbonate are necessary to release these residues. A small amount of organic co-solvent is necessary to help release residues of the more hydrophobic sulfonylureas from soils which contain high levels of organic matter. The resulting extract is concentrated and purified using a polymer resin-based SPE cartridge, which has been found to be very effective in removing most soil-related co-extractives from the sample. An additional liquid–liquid partitioning into ethyl acetate removes polar compounds that may interfere with the LC/MS/MS analysis.

2.3.3 Description of method

Soil (25 g) is extracted with 2 × 100 mL of methanol–0.1 M ammonium carbonate (1 : 9, v/v) solution using a wrist-action shaker. A refrigerated centrifuge set at 4 °C and 10 000 rpm is recommended to separate the supernatant from the solids after each extraction. The combined supernatants are charged onto an Oasis HLB SPE cartridge (1-g/20-mL, Waters) which has been preconditioned with 25 mL of methanol followed by 25 mL of the methanol–0.1 M ammonium carbonate (2 : 1, v/v) extracting solution. The sample container is rinsed with 10 mL of water, and the rinsate is passed through the cartridge. The cartridge is allowed to go dry only after the water wash has passed through and is then washed with 10 mL of hexane. The cartridge is air-dried under vacuum for 5 min and eluted with 10 mL of 1 M ammonium hydroxide–acetonitrile (1 : 19, v/v) followed by 5 mL of ethyl acetate. The cartridge is only allowed to go dry after all the ethyl acetate has passed through. The combined eluates are evaporated to approximately 1 mL under a stream of nitrogen at 25–30 °C. Then, 2 mL of water are added, and evaporation is continued until the volume is reduced to 1.8 mL, after which 10 μL of acetic acid are added. [Note: if tribenuron methyl or other extremely acid-sensitive sulfonylureas are to be analyzed, use 2 mL of pH 5.5, 5 mM ammonium acetate solution instead of water, and do not add any acetic acid. This alternative will work for all sulfonylureas except the most polar sulfonylurea tested (nicosulfuron).] In either case, the 1.8 mL of aqueous sample is extracted with 2 × 5 mL and 1 × 2 mL of ethyl acetate through vortex mixing. Centrifugation is used to help separate the layers, and additional acetic acid (if originally used) is not added after each extraction. The combined ethyl acetate layers are evaporated to dryness under a stream of nitrogen at 25–30 °C. Just prior to LC/MS/MS analysis, the sample is reconstituted in 0.3 mL of methanol and diluted to 3.0 mL with pH 6.5, 5 mM ammonium acetate.

2.4 Water

2.4.1 Nature of the residue

Hydrolysis rates of sulfonylurea herbicides in water are heavily dependent upon pH. In general, acidic conditions promote faster hydrolysis, usually by cleavage of the sulfonylurea bridge. Neutral to alkaline conditions favor the compounds existing in

their anionic forms, where they are generally more stable. However, a small number of sulfonylureas undergo *ipso*-rearrangement and bridge contraction at alkaline pH as mentioned above. Hydrolysis (and photolysis) products of sulfonylurea herbicides do not exhibit any herbicidal activity and have been shown to have no other toxicological or ecotoxicological risks. Therefore, the definition of the residue in drinking and surface waters only includes the parent compounds. The LOQ of methods for the determination of sulfonylurea herbicides in drinking waters was established based on European Union (EU) drinking water guidelines,[28] which require analytical methods that can measure levels down to 0.1 $\mu g\,L^{-1}$ (ppb). The method described below has an LOQ of 0.05 $\mu g\,L^{-1}$. In the case of surface waters, the methods must be sensitive enough to determine the no observable effect level (NOEL) derived from the most relevant ecotoxicological studies. In the case of herbicides, the appropriate studies to evaluate would be those conducted on nontarget aquatic plant species, usually algae and *Lemna gibba*. The results of these studies using various sulfonylureas indicate that 0.05 $\mu g\,L^{-1}$ is a sufficiently low LOQ to meet surface water method requirements.

2.4.2 Rationale for method

The method for water is a simplified version of the soil method described above. The water sample is adjusted to pH of 6.5, concentrated, and purified using a similar SPE procedure to that employed in the soil method. The additional cleanup with the ethyl acetate partitioning is not necessary.

2.4.3 Description of method

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A water sample (50 mL) is adjusted to pH of 6.5 by addition of 0.5 mL of 1.0 M ammonium acetate. (Note: for brackish water, the pH is adjusted to 6.0–6.2 by addition of dilute acetic acid, and the sample is diluted with an equal volume of purified water.) A polymer-based ENV SPE cartridge (0.5-g/6-mL, Part No. 952493; Varian, Harbor City, CA, USA) is preconditioned with 10 mL of methanol followed by 10 mL of 10 mM ammonium acetate. The water sample is loaded onto the SPE cartridge at a flow rate of 2–5 mL min^{-1}. Just before the entire water sample has passed through the cartridge, the sample container is rinsed with 10 mL of purified water, and the rinsate is added to the cartridge. The cartridge is air-dried under vacuum for 5 min and rinsed with 10 mL of hexane. The cartridge is air-dried under vacuum for an additional 5 min. The analytes are eluted with 15 mL of 25 mM ammonium hydroxide in methanol. The eluate is evaporated to dryness under a stream of nitrogen at 30–40 °C. Finally, the sample is reconstituted in 3 mL of 5 mM ammonium acetate for LC/MS/MS analysis.

3 Conclusions and future directions

The methods described above generally produce recoveries in the 80–110% range with relative standard deviations of 10% or less, at the stated LOQ and higher levels. The LC/MS/MS traces are generally free of interference, especially for soil and water analyses. On rare occasions, an interfering peak may be observed at one of the transitions monitored for plant-based samples, but we have never seen interference on

both of the channels. Matrix enhancement or suppression was not observed using the extractions and cleanups described above. Calibration curves are linear with negligible intercepts; therefore, either linear regression or response factors may be used for calculations.

Since sulfonylurea determinations are generally carried out at trace levels, the possibility of contamination must always be kept in mind. Samples should be kept isolated from solid analytical standards and concentrated stock solutions of standards. Only dilute standards, which are used for fortifications and chromatographic standards, should be located near the analytical samples and only for minimum amounts of time. If a confirmed response for one or more of the analytes being determined is obtained in an investigative sample (other than a deliberately fortified sample or a sample that was known to have been recently treated with the analyte), the possibility of contamination must be ruled out before a positive result can be reported. If a reliable control sample is not available, a reagent blank (prepared by taking the specified amount of extracting solution or purified water through the entire procedure) should be run. If analyte responses are present in the reagent blank, the equipment used to prepare the sample should be thoroughly cleaned (dilute bleach solutions are excellent for this purpose) and checked by preparation of additional reagent blanks before repeating the analysis. The previous data should be discarded as false-positive results.

At the present time, LC/MS/MS with triple-quadrupole instruments is the analytical method of choice for the determination of residues of sulfonylurea herbicides. We can expect to see improved triple-quadrupole instrumentation become more available and affordable as time passes, so that more analytical laboratories will have this capability. Time-of-flight (TOF) instrumentation may also play an increasingly important role in sulfonylurea analysis. Even though the metabolites are innocuous, stricter regulatory requirements may mandate that they be monitored, and LC/MS/MS is the method of choice for these compounds also.

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References

1. E.M. Beyer, M.J. Duffy, J.V. Hay, and D.D. Schlueter, 'Sulfonylureas,' in "Herbicides: Chemistry, Degradation and Mode of Action," ed. P.C. Kearney and D.D. Kaufmann, Marcel Dekker, New York, pp. 117–189 (1987).
2. I. Ahmad and G. Crawford, *J. Agric. Food Chem.,* **38**, 138 (1990).
3. P. Klaffenbach and P.T. Holland, *Biol. Mass Spectrom.,* **22**, 565 (1993).

4. E.G. Cotterill, *Pestic. Sci.,* **34**, 291 (1992).
5. D.G. Thompson and L.M. MacDonald, *J. AOAC Int.,* **75**, 1084 (1992).
6. G. Vinelli, A. Vicari, and P. Catizone, *J. Agric. Food Chem.,* **41**, 742 (1993).
7. A.J. Krynitsky, *J. AOAC Int.,* **80**, 392 (1997).
8. B.M. Berger and N.L. Wolfe, *Fresenius' J. Anal. Chem.,* **356**, 508 (1996).
9. G. Dinelli, A. Vicari, and V. Brandolini, *J. Chromatogr. A,* **700**, 201 (1995).
10. M.M. Kelley, E.W. Zahnow, W.C. Peterson, and S.T. Toy, *J. Agric. Food Chem.,* **33**, 962 (1985).
11. J.F. Brady, J. Turner, and D.H. Skinner, *J. Agric. Food Chem.,* **43**, 2542 (1995).
12. E.W. Zahnow, *J. Agric. Food Chem.,* **30**, 854 (1982).
13. E.W. Zahnow, *J. Agric. Food Chem.,* **33**, 479 (1985).
14. G.C. Galletti, A. Bonetti, and G. Dinelli, *J. Chromatogr. A,* **692**, 27 (1995).
15. C.R. Powley and P.A. de Bernard, *J. Agric. Food Chem.,* **46**, 514 (1998).
16. A.K. Sarmah and R.S. Kookana, *J. Environ. Sci. Health,* **B34**, 363 (1999).
17. L.M. Shalaby, F.Q. Bramble, and P.W. Lee, *J. Agric. Food Chem.,* **40**, 513 (1992).
18. R.W. Reiser, A.C. Barefoot, R.F. Dietrich, A.J. Fogiel, W.R. Johnson, and M.T. Scott, *J. Chromatogr.,* **554**, 91 (1991).
19. R.W. Reiser and A.J. Fogiel, *Rapid Commun. Mass Spectrom.,* **8**, 252 (1994).
20. L.M. Shalaby, *Biomed. Mass Spectrom.,* **12**, 261 (1985).
21. D. Volmer, J.G. Wilkes, and K. Levsen, *Rapid Commun. Mass Spectrom.,* **9**, 767 (1995).
22. L.J. Marek and W.C. Koskinen, *J. Agric. Food Chem.,* **44**, 3878 (1996).
23. M. Rodriguez and D.B. Orescan, *Anal. Chem.,* **70**, 2710 (1998).
24. R. Bossi, B. Koppen, N.H. Spliid, and J.C. Streibeg, *J. AOAC Int.,* **81**, 775 (1998).
25. T. Cairns, 'Development of Guidelines for Detection, Confirmation and Quantitation by GC/MS, LC/MS and MS/MS,' Presented at ASMS Workshop, November 1996.
26. L.Y.T. Li, D.A. Campbell, P.K. Bennett, and J. Henion, *Anal. Chem.,* **68**, 3397 (1996).
27. T.T. Obrigawitch, G. Cook, and J. Wetherington, *Pestic. Sci.,* **52**, 199 (1998).
28. European Commission, Directorate General Health and Consumer Protection, 'Guidance Document on Residue Analytical Methods,' SANCO/825/00 rev. 6, June 20, 2000, European Commission, Brussels (2000).

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Triazine herbicide methodology

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1 Introduction/general description

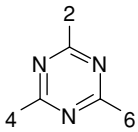
The first triazine herbicide was developed almost 50 years ago in Basel, Switzerland, in the laboratories of J. R. Geigy AG,[1,2] and since that time, more than 25 commercial products have been developed. The s-triazines can be divided into the chloro- (-azine), methoxy- (-ton), and methylthiotriazine (-etryn) groups depending on the substitution at the 2-position. The other two carbon positions in the s-triazine structure contain substituted amino groups. Asymetric triazines include metribuzin and metamitron. The structures and abbreviations for a few selected s-triazine compounds are shown in Table 1. Some of the abbreviations will be used throughout this article to simplify the identification of these compounds in the text and other tables. The nomenclature used for the common names is atypical in that the names are derived from the parent compounds (e.g., atrazine) and the group cleaved from the molecule during degradation or metabolism (e.g., deethylatrazine wherein the ethyl group was cleaved from the molecule).

Chemical and physical data for commercially important triazines are shown in Table 2.[3–5] These compounds have low vapor pressures and relatively high melting points (88 °C for ametryn to 227 °C for simazine). They are generally white crystalline solids at room temperature with water solubilities ranging from 5 to 1220 mg L^{-1} depending on the substitutent at the 2-position, decreasing in the order methoxy- $\gg$ methylthio- $>$ chloro-.[6] Their solubilities increase at pH levels near their respective pK_a values owing to strong protonation reactions. The dialkylaminotriazines are weak bases in aqueous solution with the basicity decreasing in the order methoxy- $>$ methylthio- $>$ chloro-substituted triazines. The octanol–water partition coefficients of several triazine compounds were measured using liquid chromatography (LC),[7] and the log K_{ow} values obtained compared well with previously reported literature values, e.g., atrazine 2.46, simazine 2.11, DEA 1.39, DIA 1.01, DACT 0.11, HA 0.76 (see Figure 1 for structures). Capillary zone electrophoresis was used to determine the pK_1, pK_2, and pI values for 12 hydroxytriazines.[8]

In general, triazines are pre- and post-emergence selective herbicides particularly effective on annual and perennial broadleaf and grassy weeds in corn, sorghum, cotton, soybeans, sugar cane, and a host of other fruit and cereal crops.[9] Some have anti-fungicidal properties (e.g., anilazine), and some (e.g., simazine) can be used for

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Table 1 Structures of selected triazine compounds



Triazine	2	4	6	Abbreviation
Anilazine	−Cl	−Cl	$-NHC_6H_4$ (aromatic)	AN
Atrazine	−Cl	$-NHC_2H_5$	$-NHC_3H_7$ (iso)	ATZ
Simazine	−Cl	$-NHC_2H_5$	$-NHC_2H_5$	SIM
Chlorazine	−Cl	$-N(C_2H_3)_2$	$-N(C_2H_3)_2$	CH
Cyromazine	$-NHC_3H_5$ (cyclo)	$-NH_2$	$-NH_2$	CR
Cyanazine	−Cl	$-NHC(CN)(CH_3)_2$	$-NHC_2H_5$	CY
Propazine	−Cl	$-NHC_3H_7$ (iso)	$-NHC_3H_7$ (iso)	PRZ
Metribuzin				MB
Terbuthylazine	−Cl	$-NHC_2H_5$	$-NHC(CH_3)_3$	TER
Trietazine	−Cl	$-N(C_2H_3)_2$	$-NHC_2H_5$	TRI
Ametryn	$-SCH_3$	$-NHC_2H_5$	$-NHC_3H_7$ (iso)	AME
Prometryn	$-SCH_3$	$-NHC_3H_7$ (iso)	$-NHC_3H_7$ (iso)	PME
Simetryn	$-SCH_3$	$-NHC_2H_5$	$-NHC_2H_5$	SIY
Terbutryn	$-SCH_3$	$-NHC_2H_5$	$-NHC(CH_3)_3$	TEY
Prometon	$-OCH_3$	$-NHC_3H_7$ (iso)	$-NHC_3H_7$ (iso)	PRM
Deethylatrazine	−Cl	$-NH_2$	$-NHC_3H_7$ (iso)	DEA
Deisopropylatrazine	−Cl	$-NHC_2H_5$	$-NH_2$	DIA
Deethyldeisopropylatrazine	−Cl	$-NH_2$	$-NH_2$	DACT
Deethylterbuthylazine	−Cl	$-NH_2$	$-NHC(CH_3)_3$	DET
Hydroxyatrazine	−OH	$-NHC_2H_5$	$-NHC_3H_7$ (iso)	HA
Hydroxysimazine	−OH	$-NHC_2H_5$	$-NHC_2H_5$	HSIM
Hydroxyterbuthylazine	−OH	$-NHC_2H_5$	$-NHC(CH_3)_3$	HTER
Hydroxypropazine	−OH	$-NHC_3H_7$ (iso)	$-NHC_3H_7$ (iso)	HPRZ
Deisopropylhydroxyatrazine	−OH	$-NHC_2H_5$	NH_2	HDIA
Deethylhydroxyatrazine	−OH	$-NH_2$	$-NHC_3H_7$ (iso)	HDEA
Deethyldeisopropyl-hydroxyatrazine	−OH	$-NH_2$	NH_2	HDACT

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Table 2 Chemical and physical data for commercially important triazine compound (from Refs. 3–5)

Compound	Melting point (°C)	Solubility in water at 20–25 °C (mg L^{-1})	pK_a	Oral LD_{50} (mg kg^{-1})
Ametryn	88–89	185	3.93	965 (mice); 1100 (rat)
Anilazine	159–160	Insoluble	—	>5000 (rat)
Atrazine	171–174	33	1.68	1750 (mice); 3080 (rat)
Chlorazine		10	—	
Cyanazine	168–169	171	1.1	380 (mice); 182 (rat)
Cyromazine	219–222	1220	—	
Metribuzin	126–127	1050	—	1090–1206 (rat)
Prometon	91–92	750	4.28	2980 (rat)
Prometryn	118–120	48	1.0	3750 (rat)
Propazine	212–214	9	1.85	>5000 (rat)
Simazine	226–227	5	1.65	5000 (rat)
Terbuthylazine	177–179	9	1.12	2160 (rat)
Simetryn	81–83	450	—	
Turbutryn	104–105	58	—	

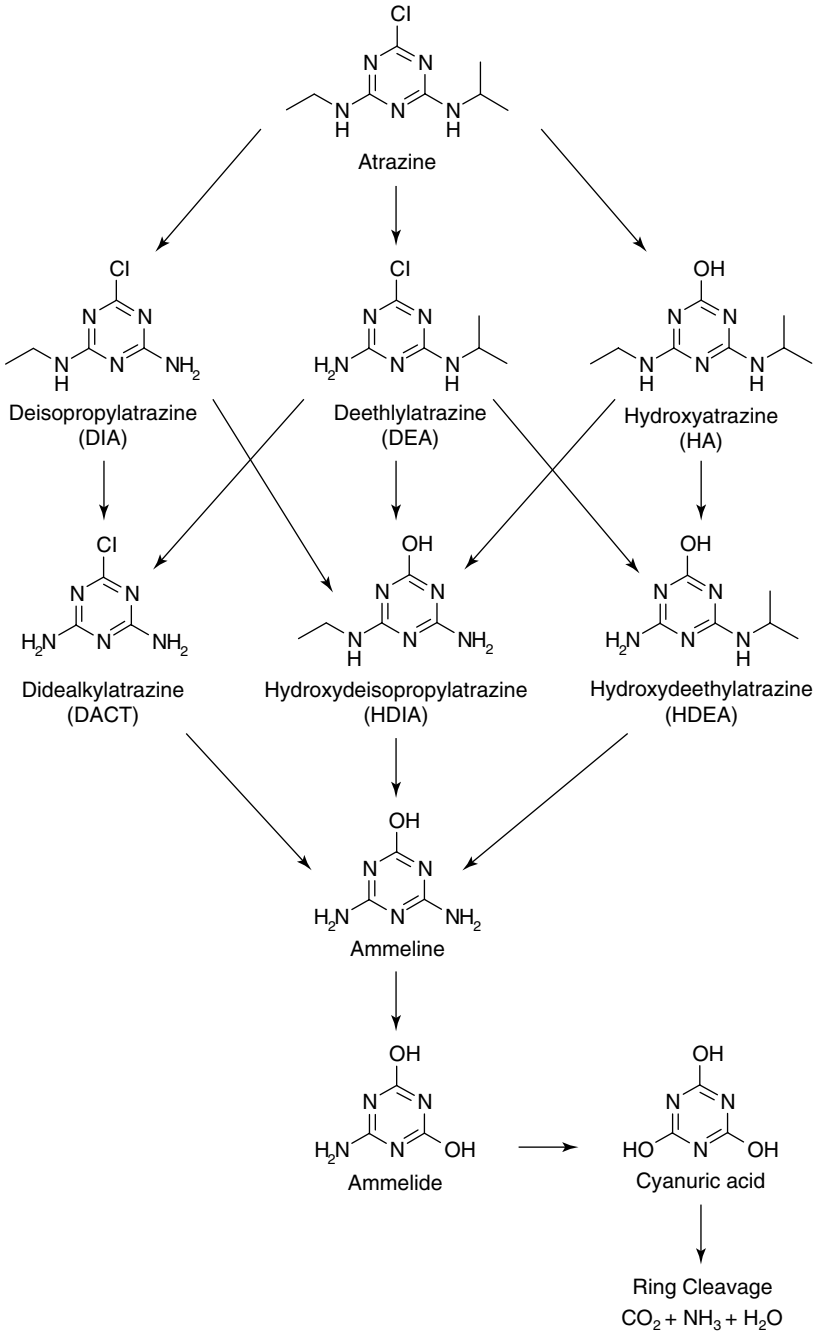


Figure 1 Pathways for the degradation of atrazine (from Ref. 23)

total nonselective weed control. Their herbicidal activity is directed primarily against seedling weeds. They are readily absorbed by the plant roots and transported to the tips and margins of the leaves where they interfere with the photosynthesis enzyme system.[10]

Of the ca 20 different classes of herbicides, the triazine class of compounds is among the most widely used worldwide.[11] Atrazine and simazine are among the most often monitored and studied compounds in groundwater, surface water, and soil. Triazines metabolize extensively in plants and animals[12] and degrade in the environment via chemical and physical processes[13] and microbial degradation.[14] Major metabolic and degradation reactions include dealkylation, oxidation, dechlorination, and hydrolysis reactions to form the chlorodealkylated and eventually the hydroxytriazine products. For s-triazines, continued degradation eventually leads to the formation of cyanuric acid[15] and, in many cases, further dealkylation and opening of the ring (mineralization) to form carbon dioxide and ammonia.[16] A general depiction of the various metabolic/degradative pathways for atrazine is shown in Figure 1. As the metabolism and/or degradation of the parent compounds proceeds, the subsequent and succeeding products increase in polarity, which increases their water solubility and decreases their ability to adsorb to soil. The overall effect is an increase in mobility and a propensity to leach into ground water.[17] For the purpose of simplifying the discussion in this article, all the metabolites and degradates of parent triazine compounds will be referred to collectively as 'degradates' regardless of their chemical origin and the reaction pathways that resulted in their presence in the environment.

The European Economic Community (EEC) established a priority list of pesticides with a maximum admissible concentration (MAC) of 0.1 $\mu g\,L^{-1}$ (ppb) per pesticide in water intended for human consumption.[18] The list contains compounds that have a probability of leaching and includes the triazines: atrazine, simazine, cyanazine, prometryn, terbuthylazine, and terbutryn. The maximum permissible level for compounds not on the priority list is 0.5 $\mu g\,L^{-1}$. In the USA, the Office of Drinking Water (ODW) of the Environmental Protection Agency (EPA) established drinking water regulations and a health advisory level (HAL) for individual pesticides. The HAL is not a legally enforceable federal standard but serves as technical guidance to assist federal, state, and local officials. However, the maximum contaminant level (MCL) is the highest level of contaminant that is legally allowable in drinking water, and this standard is enforceable under law. For example, the MCLs for atrazine and simazine are 3 and 4 $\mu g\,L^{-1}$, respectively.[19] At present, MCLs have not been established for the degradates of atrazine or simazine, but summing the concentrations of the parent and their respective degradates, regardless of their toxicological significance, is under consideration.

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A plethora of methods developed for the determination of triazine compounds in water, soil, crops, biological fluids, etc., have been reported in the literature, and several excellent reviews are available for the interested reader.[20–23] More method papers are published on the determination of triazines in water than for all other sample matrices combined (water $\gg$ soil $>$ crop). The majority of the water method reports relate to the determination of parent triazine compounds plus compounds from one or more other chemical classes of pesticides (e.g., phenoxy acids, carbamates, phenylureas, acetanilides, acetamides, organophosphorus compounds, etc.) for generalized multi-residue screening or monitoring purposes. Addressed in other more selective

studies are the determination of parent triazine compounds only or the determination of parent triazines and some of their degradation products. The measurement uncertainties associated with methods for the determination of triazine compounds in groundwater were reviewed and discussed.[24]

2 Analytical methodology for water samples

The most widely employed techniques for the extraction of water samples for triazine compounds include liquid–liquid extraction (LLE), solid-phase extraction (SPE), and liquid–solid extraction (LSE). Although most reports involving SPE are off-line procedures, there is increasing interest and subsequently increasing numbers of reports regarding on-line SPE, the goal of which is to improve overall productivity and safety. To a lesser extent, solid-phase microextraction (SPME), supercritical fluid extraction (SFE), semi-permeable membrane device (SPMD), and molecularly imprinted polymer (MIP) techniques have been reported.

2.1 Water sample preparation

2.1.1 Liquid–liquid extraction

LLE has been used for decades and is one of the earliest procedures used for the extraction of pesticides from water samples. Several official methods of analysis still rely on LLE, including EPA Method 507 in which dichloromethane is used as the extraction solvent; the method is applicable to the extraction of a wide range of pesticide classes including certain triazines.[25] The organic solvent is concentrated followed by a solvent switch to methyl *tert*-butyl ether prior to injection. Typical LLE sample volumes are <1 L, but sample volumes as large as 120 L have been reported for ultra-trace level work.[26] Recently reported multi-residue methods relying on LLE attest to the continued use and effectiveness of these techniques,[27–35] most of which employ dichloromethane as the extraction solvent. The multi-residue LLE of several triazine compounds and their degradates, including DACT, was reported.[36] Although the extraction and analysis of DACT were not addressed in most reports, this polar degradate can be conveniently isolated separately using SPE.[37]

The LLE technique is undoubtedly labor intensive and costly owing to the expense associated with the use of large volumes of organic solvents and their associated disposal costs when compared with other and more recent water sample preparation procedures (e.g., SPE). LLE is difficult to automate, and complications arise due to varying analyte extraction efficiencies and the formation of emulsions.[38] However, the cost must be weighed against the number of analytes extracted and available for analysis and, in some cases, LLE may still be competitive regarding cost per analyte per sample. These factors must be weighed in the light of the goals and overall objectives of a particular study. In some cases, micro-LLE may be applicable, which reduces the volume of organic solvent required to perform the extraction.[39,40]

2.1.2 Solid-phase extraction

SPE in cartridge or disk form is a rugged and reliable technique used in water analysis and is applicable to numerous classes of organic compounds including the

triazines. The tremendous number of papers published in the last 5–7 years devoted to SPE is a testament to its popularity, usefulness, and general applicability for the preparation of samples for analysis in the environmental, pharmaceutical, clinical, and food sectors. Currently, more than 50 companies manufacture SPE products,[41] and part of the impetus in the last few years to employ SPE techniques was to reduce the significant volumes of solvents required when preparing samples for analysis using LLE. A rigorous discussion of the theory and practice of SPE is beyond the scope of this single article, but the interested reader is directed to the text of Thurman and Mills[42] and reviews by Hennion *et al.*[43] and Hennion.[44]

In SPE, an analyte can be isolated from an aqueous sample or extract using reversed-phase, normal-phase, or ion-exchange modes, depending on whether the analyte is nonpolar, polar, or ionic, respectively. A size-exclusion mode can be obtained using silica gel of wide pore size (275–300 Å), and mixed-mode sample preparation can be employed for multi-residue purposes. Sometimes mixed-mode sample preparation occurs inadvertently owing to the presence of non-end-capped polar functional sites, and this can be advantageous when trying to retain analytes of widely varying polarity such as parent triazines and their degradates. Cartridges or syringe barrels (with solvent reservoir) are typically constructed of polypropylene or polyethylene containing 50 mg to 10 g of packing material (traditionally 40-μm particle size silica gel, 60-Å pore size) to which various functional groups are chemically bonded depending on the desired mode of analyte retention. Since the advent of SPE in the mid-1970s,[22,45,46] several new sorbent types have been developed for the extraction of compounds from aqueous samples. Examples include octadecyl (C-18), octyl (C-8), and phenyl for reversed-phase, cyano, amino, diol, silica gel, and Florisil for normal-phase, and quaternary amine (anion) and aromatic sulfonic acid (cation) for ion-exchange methods. Copolymeric (e.g., styrene–divinylbenzene) and activated carbon packing materials [graphitized carbon black (GCB)] were later introduced, and these are characterized by larger specific surface areas and higher carbon loading, resulting in higher capacities. These also exhibit improved capability for retaining the more polar analytes (e.g., DACT). The capacity of the sorbent milligrams per gram of analyte that may be sorbed is a function of the phase chemistry and the weight percentage of carbon present (carbon loading). For example, typical C-18, C-8, and C-2 packing materials contain carbon loadings of 17, 14, and 5%, respectively. The cartridges are generally attached to vacuum manifolds to draw the sample and eluting solvents through the packing material, although positive pressure is sometimes used to push the solvents through the cartridge.[42] In some applications, C-8 cartridges provide clearer chromatographic profiles than C-18 cartridges.[47]

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The membrane disks used in SPE range in diameter from 4 to 90 mm, although 47 mm seems to have become the 'standard', and a height of about 0.5 mm. The first disks consisted of polytetrafluoroethylene (PTFE or Teflon) fibrils into which were embedded 8–12-μm sized particles of packing material. About 90% of the weight of the disk was due to the sorbent particles. More rigid fiber-glass-based disks were later introduced. Disks are also available in syringe-barrel format. The early disks, like the cartridges, were primarily C-18, but now several phases are available in all three modes of analyte isolation. The disk is usually supported on special glassware using a Kel-F support, clamp, and reservoir, and the whole assembly is attached to an Erlenmeyer flask containing a side-arm connection for a vacuum source. The primary advantage of the disk configuration is rapid mass transfer due to the greater specific surface area and

higher sample flow rates. Channeling problems such as those encountered in cartridge SPE are minimal using disks. In spite of these advantages, recoveries on C-8 and C-18 cartridges tend to be higher, in general, than those obtained on C-8 and C-18 disks.[48]

The SPE technique, using cartridges or disks, basically consists of four steps: (1) conditioning the sorbent, (2) loading the sample, (3) elution of interferences, and (4) elution of the analyte(s). In step (1), the SPE disk or cartridge is conditioned with an appropriate solvent to wet the packing material, solvate the functional groups of the sorbent, and remove air. This is usually followed by the addition of water or buffer to activate the cartridge such that the sorption mechanism works properly for aqueous samples. Care must be taken not to allow the sorbent to dry. If required, the eluting solvent, e.g., methanol, can be added during conditioning to remove interfering impurities that may be in the packing materials, e.g., benzylsulfonic acid.[49] If this cleanup step is required, the sorbent must be prepared again for sample addition by adding water or buffer. In step (2), a sample volume of 1–1000 mL is added to the cartridge via gravity, pumping, or vacuum. The loading rate must not exceed the kinetics of the mechanism of retention (van der Waals interaction, hydrogen bonding, dipole–dipole forces, size exclusion, and ion exchange) between the analyte and the sorbent. Thus, the rate at which the sample is allowed to pass through the disk or cartridge is dependent on the nature of the sorbent and the targeted analyte to be retained. In step (3), interferences are removed from the interstitial spaces of the cartridge by rinsing the sorbent with an appropriate solvent system (aqueous or aqueous/organic mixture). In the last step (4), the analyte is removed from the disk or cartridge with an appropriate volume of elution solvent specifically chosen to disrupt the interaction between the analyte and the sorbent. Ideally, the eluting solvent should remove as little as possible any other substances sorbed on the cartridge or disk.[42] The term digital liquid chromatography was coined to describe this on/off mechanism of SPE.[50] Solvent reduction (e.g., rotary evaporation, nitrogen evaporation, etc.) is employed if further analyte enrichment is required prior to injection and analysis.

Several SPE procedures reported for triazine compounds are summarized in Table 3, and some are applicable to the quantitative extraction of parent triazine compounds.[51–62] The SPE sorbents employed include C-18, GCB, and DVB, and for the most part, acceptable recoveries (70–120%) were obtained for parent triazine compounds. For example, a 500-mg Envi C-18 SPE cartridge was conditioned with 10 mL of methanol and equilibrated with 10 mL of Milli-Q water. A 250-mL water sample was loaded on to the column after adjusting the sample pH to <2 with phosphoric acid. The cartridge was dried under vacuum for 5 min, and the analytes were eluted with 1 mL of methanol. The solvent was evaporated under a gentle stream of nitrogen, and the sample was reconstituted in 0.5 mL of mobile phase. Analysis was performed using LC/UV detection at 230 nm.[63] The recoveries obtained were 76% for atrazine, 78% for simazine, 81% for cyanazine, and 97% for ametryn. In another report, a 50-mm Speeddisk bonded with 750 mg of C-18 was rinsed with 5 mL of dichloromethane and then conditioned with 10 mL of methanol and 10 mL of ultrapure water. A 1-L volume of water sample was acidified to pH 2 using 2 mL of 6 N HCl , and 5 mL of methanol were added to improve the extraction of nonpolar and slightly polar compounds. The sample was passed through the disk at 200 mL min^{-1}. The analytes were eluted using 10 mL of dichloromethane followed by 10 mL of dichloromethane containing 100 μL of n-dodecane as a 'keeper' (to minimize the

Table 3 Summary of solid-phase extraction techniques applied to the preparation of water samples for the determination of triazine pesticides

Analyte(s)	Matrix[a]	Sample preparation[b]	Instrumentation[c]	Recovery data summary	Ref.
ATZ, CY, SIM, TER	GW, RW, PW	Carbograph 4 GCB	LC/MS	97–102%	51
ATZ, MET		Oasis HLB, C-18	LC/DAD	98–100% (ethyl acetate)	52
ATZ, SIM, AME, PME, TER	RW	Bond-Elut	GC/ECD and GC/MS	70–91%	53
ATZ, SIM, PRZ, PME, CY	RW, SW, SEW	Empore C-18 disk	GC/FTD and GC/MS	40–105%	54
ATZ, SIM, PRZ, AME, PME, TER	Natural waters	Oasis HLB	CZE	83–114%	55
ATZ, SIM, CY, AME, PRZ, PME, TER	DIW	C-18 cartridge	MECC	91–116%	56
ATZ, MET	SW	GCB	LC/UV	94–95%	57
ATZ, PRM, TER	SW	SPME (65-μm CW–DVB-coated fiber)	GC/NPD		58
ATZ, SIM	GW	XAD-2 or C-18	GC/NPD, GC/MS	74–85%	59
ATZ, SIM, AME, PRZ, PRM, PME, SIY, TER	GW	SPME (using 100-μm PDMS)	GC/NPD, GC/MS		60
ATZ, MET, PRM, SIM, TER	SW	C-18 bonded silica	GC/ECD, GC/NPD, GC/MS		61
ATZ	DIW	C-18 47-mm disks	GC/MS	80–110%	62
ATZ, SIM, PRZ, TER, DEA, DIA	SW	SAX and C-18, double disk	LC/DAD	101–110% ATZ 25% DIA; 85% DEA	67
ATZ, DEA, SIM	SW	C-18 PS–DVB	GC/MS, GC/NPD, GC/ECD		68
ATZ, DEA, DIA, DIHA, DACT, DEHA, HA	GW, SW	Carbograph 4 GCB	LC/MS	80–101%	69
ATZ, DIA, DEA, SIM, CY	SW	C-18 Empore disk	LC/UV and LC/MS	80–125% (except DIA and DEA, <9%)	70
HA, HDEA, HDIA	SW	SCX	LC/UV, LC/MS		71
ATZ, DEA, DIA, HA	Run-off	Tandem SPE, C-18 and SCX	LC/DAD and LC/MS	96–99% ATZ, DEA, DIA on C-18, 78–103% DACT, HA on SCX	72
ATZ, AME, DEA, DIA, CY, MET, PM, PRZ, SIM	SW	Carbograph B GCB	GC/NPD, LC/MS	51–84%, 5%, MET	73, 74
ATZ, DEA, DIA, DET, CY, PRZ, SIM, TER	SW	PS–DVB copolymer	LC/DAD	94–109%	75
HA	DIW, GW	GCB	FAB/MS/MS	85% at 5 ng L^{-1} and 94% at 500 ng L^{-1}	78
HA, DEHA, DIHA	Creek water	SCX	LC/UV	87–90% at 5 μg L^{-1}	79

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Table 3—*Continued*

Analyte(s)	Matrix[a]	Sample preparation[b]	Instrumentation[c]	Recovery data summary	Ref.
ATZ, HA, HDEA, HDIA	SW, run-off, wastewater	GCB	LC/DAD	92–101%	80
Hexazinone and 5 metabolites	GW	Envi-Carb GCB	CE	79–100% 30–120% for metabolites	81
ATZ, DEA, DIA, SIM, TER, AME, PME, desmetryn, SIY, TEY, metamitron, terbumeton	DIW	Three tandem C-18 cartridges	GC/ITD	75–120% for two-thirds of compounds 12–50% for the more polar compounds (e.g., DEA, DIA)	82
HA, HSIM, HTER, HDACT	GW	1 g of C-18-modified silica	LC/DAD	121% for HA, 107% for HTER, and 37% for HSIM. Recovery not reported for HDEDIA	83
ATZ, HA	Run-off, DIW	Carbon black cartridge	LC/ESI-MS	108%	84
ATZ, DEA, DIA, SIM, PRZ, TER,	Run-off, RW	GCB and derivatization	GC/MS	85 and 95% for DIW and RW/SW, respectively.	85
ATZ, TER, DEA, DIA, DET, HA, HTER, DEHA, DIHA, HDACT (ameline)	RW, PW	SDB	GC/MS and CZE/UV, MECC	>80% for HA, Hter, DEHA 30% for DIHA. All others quantitatively recovered	86
ATZ, DEA, DIA, HA	SW, SEW	GCB disks, LiChrolut EN, aminopropyl cartridges	LC/ESI-MS/MS	77–88% using LiChrolut EN	87
ATZ, SIM, HA, DIA, DEA, PROPZ, PROME	Mineral, DW	C-18 disk and two PS–DVB disks	MEKC	74–102% for two PS–DVB disks	88
ATZ, DEA, DIA, DACT, HA	GW	ENVI-carb GCB	GC/MS after derivitization	77–107%	89
ATZ, SIM, DEA, DIA, TER	SW	Oasis	LC/APcI-MS	76–96% at 2 $\mu g\,L^{-1}$	90
ATZ, HA	DW	Envi-18	LC/ISP-MS	97% for ATZ, HA not recovered	91
DEA, DIA	DIW, SW, GW, run-off	Tandem C-18	GC/MS	105–117% at 0.5 to 1.0 $\mu g\,L^{-1}$	92

[a] GW = groundwater; SW = surface water; PW = potable water (drinking water); SEW = sea water; DIW = deionized water; RW = rain water.

[b] GCB = graphitized carbon black; SPME = solid-phase microextraction; PDMS = polydimethylsiloxane; PS = polystyrene; DVB = divinylbenzene; SDB = styrene–divinylbenzene.

[c] LC/MS = liquid chromatography/mass spectrometry; LC/DAD = liquid chromatography/diode-array detection; GC/ECD = gas chromatography/electron capture detection; GC/MS = gas chromatography/mass spectrometry; CZE = capillary zone electrophoresis; MEKC = micellar electrokinetic chromatography; LC/UV = liquid chromatography/ultraviolet; GC/NPD = gas chromatography/nitrogen–phosphorus detection; FAB/MS/MS = fast atom bombardment tandem mass spectrometry; CE = capillary electrophoresis; GC/ITD = gas chromatography/ion-trap detection; LC/ESI-MS = liquid chromatography/electrospray ionization mass spectrometry; CZE/UV = capillary zone electrophoresis/ultraviolet; LC/ESI-MS/MS = liquid chromatography/electrospray ionization tandem mass spectrometry; LC/APcI-MS = liquid chromatography/atmospheric pressure chemical ionization mass spectrometry; LC/ISP-MS = liquid chromatography/ionspray mass spectrometry; MECC = micellar electrokinetic capillary chromatography; SAX = strong anion exchange; SCX = strong cation exchange.

loss of the more volatile compounds). The eluate was evaporated to 1 mL and analyzed by GC/MS. Recoveries of 89–105% were obtained for atrazine, simazine, propazine, and terbuthylazine over a fortification range of 200–1000 ng L^{-1}.[64] However, these applications were for parent triazines only since the overall goal was to design the procedure in such a manner as to extract analytes from several classes of compounds. Thus, the nonpolar sorbents performed well for the recovery of parent triazine compounds, but the more polar degradates were not retained (or studied). For example, owing to low breakthrough volumes, the more polar metabolites such as DEA, DIA, and HA could not be quantitatively recovered.[65,66]

Several reported SPE procedures for triazine compounds and some of their degradation products in water are also shown in Table 3.[67–92] Although the C-18 SPE mode is still frequently employed, the introduction of additional sorbents allowed the extraction of many of the polar degradates of the triazine compounds. The use of GCB, PS–DVB, SAX, SCX, and various combinations to obtain mixed mode retention of the desired analytes on the cartridge or disk provided quantitative recovery in many cases. Recoveries of 74–102% for atrazine, simazine, HA, DIA, DEA, propazine, and prometryn at the 1 μg L^{-1} concentration level in purified water were obtained when using two PS–DVB disks.[88] The 47-mm disks were conditioned with 2 × 10 mL of methanol and 2 × 10 mL of water followed by loading a 1-L water sample. The disks were then washed with 5 × 2 mL of water and vacuum dried, and the analytes were eluted using 6 × 2 mL of methanol. The eluent was evaporated to dryness, the residue was reconstituted in 1 mL of 10 mM sodium borate buffer, and the final fraction was filtered through a 0.45-μm PTFE filter prior to injection (the final analysis was performed using MEKC). The recoveries for DIA and DEA were unacceptable at 22–61% when using one or two in-tandem C-18 disks or when using only one PS–DVB disk. Recoveries of 76–102% at the 0.10 and 0.50 μg L^{-1} concentration levels were obtained when the matrix was mineral water, but DEA was not detected in tap or well water. This was presumed to be due to water hardness (Ca^{2+} and Mg^{2+}) and the presence of humic and fulvic acids. In another application, recoveries of 77–107% were obtained for atrazine, DEA, DIA, HA, and DACT at the 0.8 and 8 μg L^{-1} concentration levels when using an Envi-Carb 250-mg GCB cartridge.[89] The cartridge was conditioned with 6 mL each of dichloromethane, dichloromethane–methanol (7 : 3, v/v), methanol, and water. A 100–175-mL volume of sample was then pumped through the cartridge at a rate of 2–3 mL min^{-1} followed by drying under vacuum to remove interstitial water. The analytes were eluted with 3 mL of ethyl acetate and then 8 mL of dichloromethane–methanol (7 : 3, v/v). The two eluents were collected separately and re-combined after drying the ethyl acetate fraction through a 1-g bed of sodium sulfate. An internal standard was added, and a solvent switch to acetonitrile was performed prior to attaining a final fraction volume of 100 μL. In this method, the analytes were derivatized for analysis by GC/MS. The use of SDB, OASIS, Envi-Chrom, and Envi-Carb sorbents appears to be promising for multi-residue methodology including the determination of triazine compounds and their degradates.[85] A comparison study using PS–DVB and GCB SPE for the extraction of triazines and their degradates from water was reported.[93] The GCB cartridges (Envi-Carb) were superior to the PS–DVB cartridges (LiChrolut EN) for the extraction of the more polar degradates.

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2.1.3 On-line SPE

On-line solid-phase extraction/gas chromatography (SPE/GC) was first demonstrated in 1987,[94] and since then, considerable effort has been expended to improve the coupling between SPE and gas chromatography (GC). Later work demonstrated the advantages of the on-line coupling of SPE with LC.[95] Since methanol, acetonitrile, and water are LC compatible, removal of all the residual water from the SPE cartridge or disk is not needed; plus, environmental and biological samples are already primarily aqueous. Trace enrichment of analytes via SPE and analysis (GC/ECD, GC/NPD, LC/UV, LC/DAD, LC/MS, etc.) can be automated for the monitoring of a wide range of pesticides in water (e.g., the Prospekt system from Spark Holland, Emmen, The Netherlands) using switching valves. For example, in on-line soild-phase extraction/liquid chromatography (SPE/LC), the enrichment of trace components is obtained using a solvent delivery system to purge, wash, and activate the SPE column prior to loading the sample. The enriched components are then desorbed from the SPE column directly into the analytical column using a suitable mobile phase. The SPE cartridge (or precolumn) should be pressure-resistant and have dimensions that are compatible with those of the analytical column. The goal is to transfer the concentrated sample components to the analytical column in a narrow profile to minimize band broadening during the separation. Columns used in LC typically contain 3–10-μm particle sizes, but the particle sizes used in on-line SPE are typically 15–40 μm to allow higher sampling rates. Some of the advantages reported for on-line monitoring include no sample manipulation between preconcentration and analysis, no loss or contamination risk, more accurate results, and lower limits of detection. A disadvantage of on-line versus off-line analysis is the extraction of numerous other sample components that may, in some cases, cause severe interference for the analyte(s) of interest. The requirements for on-line SPE and LC were reviewed,[96] and SPE sorbent comparisons for the analysis of atrazine and simazine were investigated.[97] In recent years, the robustness of the on-line technique was demonstrated, and the number of reported applications has significantly increased. On-line SPE use in a routine testing laboratory environment was evaluated.[98]

Summarized in Table 4 are several recent reports regarding on-line SPE/LC and SPE/GC.[99–109] Recoveries of 92–99% were obtained for atrazine, simazine, ametryn, and prometryn in water samples at the 1 $\mu g\,L^{-1}$ concentration using on-line SPE/GC/MS (selected-ion monitoring mode). A 10 × 2-mm i.d. precolumn packed with PS–DVB (PLRP-S, 20-μm particle size) was used as the SPE cartridge, and three six-port valves and an LC pump were employed during the sample preparation process. The pump delivered sample and solvents (to clean and activate) to the precolumn, and the eluent (100 μL of ethyl acetate) was delivered by a syringe pump. The analytes were transferred from the precolumn to the gas chromatography (GC) system using a 30 cm × 0.10-mm i.d. fused-silica capillary mounted permanently to the on-column GC injector. The addition of 30% methanol to 10 mL of sample prior to loading the precolumn improved the recoveries of ametryn and prometryn. The recoveries for atrazine and simazine appeared to be unaffected by the addition of methanol at levels from 0 to 30%. In this work, various transfer operating parameters (flow rate, temperature, solvent vapor exit time, etc.) were evaluated and optimized, and the viability of the technique was demonstrated.[107]

Table 4 Summary of on-line solid-phase extraction techniques applied to the determination of triazine pesticides

Analyte(s)	Matrix[a]	Sample preparation[b]	Instrumentation[c]	Recovery data summary	Ref.
ATZ, SIM, CY, DIA, HA, DEA, TER, simetryn, sebutylazine	DW, SW	SDB (PLRP-S); 15- to 25-μm, 10 × 2 mm cartridges (Prospekt) and C-18	LC/DAD	No recovery reported; SDB better than C-18 for trapping polar degradates, good reproducibility at 1 μg L^{-1}; interferences using UV	99
ATZ, AME, TER, CY, SIM, orome, dipropetryn	DW, SW	SDB (PLRP-S); 15- to 25-μm; 10 × 2 mm cartridges (Prospekt)	LC/DAD and LC/PB-MS	77–96% for ATZ, AME, TER when sample adjusted to pH 9; 70% for ATZ when pH < 7; 74–102% for all at neutral sample pH at 1 μg L^{-1}	100
ATZ, SIM, CY, AME, PME, terbutryn	SW	Nine SDB disks in holder; 47-mm containing 500 mg PS–DVB	LC/DAD	74–92% at 4 μg L^{-1}, LOD = 0.03 μg L^{-1}	101
TER, PROPZ	SW	SDB (PLRP-S); 15- to 25-μm, 10 × 2 mm cartridge (Prospekt)	LC/APcI/ESI-MS/MS	LOD about 0.4 ng L^{-1}, Recovery data not evaluated	102
ATZ, SIM, PROPZ, TER, sebutylazine	SW	Polygosil, C-18, 10-μm	LC/FTIR	87–99% for 20-mL sample size at 5 μg L^{-1}	103
ATZ, SIM, TER, DEA, DIA	SW	PS–DVB, PRP1, 10-μm	LC/UV/ electrochemical	97–106% for parent triazines 7–71% for DEA and DIA	104
ATZ, SIM, CY, PROPZ, TER, sebutylazine	SW	5-μm C-18, 10 × 2 mm 8-μm C-18, 10 × 2 mm 10–15-μm PLRP-S, 10 × 2 mm	LC/TSP-MS/MS	LOD = 1 μg L^{-1}	105
ATZ, SIM, PROPZ, TER, DEA, DIA, desethylterbutylazine	SW	Restricted access (C-18-diol-silica), C-18, PS–DVB, LiChrolut EN	LC/DAD/LC/TSP-MS	73–94% on LiChrolut EN at 1.6 μg L^{-1} 21% on other sorbents for DIA	106
ATZ, SIM, AME, PME	SW, DW	PS–DVB (PLRP-S), 20-μm	GC/MS	92–99% when 30% MeOH added to sample prior to SPE	107
ATZ, DEA, DIA	SW	PS–DVB (Amberchrom GC-161 m, PLRP-S-10, and S-30)	LC/UV	73% DIA and DEA 74% for ATZ when using 20-mL sample size. Recovery decreases significantly for DIA with increasing sample size	108
CY, CY amide, CY acid	GW	C-18 and PLRP-S	LC/APcI-MS	84–108% at sample pH 2.5	109

[a] See footnote a to Table 3.
[b] See footnote b to Table 3.
[c] See footnote c to Table 3; LC/PB/MS = liquid chromatography/particle beam mass spectrometry; LC/APcI/ESI-MS/MS = liquid chromtography/atmospheric pressure chemical ionization/electrospray ionization tandem mass spectrometry; LC/FTIR = Fourier transform infrared; LC/TSP-MS/MS = liquid chromatography/thermospray tandem mass spectrometry; LC/TSP-MS = liquid chromatography/thermospray mass spectrometry.

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Various porous crosslinked PS–DVB beads and PLRP-S resins were modified by adding *o*-carboxybenzoyl groups to their surface[108,110] and used as precolumn sorbents (10 × 3-mm i.d.) for on-line SPE/LC/UV. The goal was to retain and determine quantitatively some of the more polar triazines such as DEA and DIA. The sorbent was activated using 2 mL of acetonitrile and 2 mL of Milli-Q water (pH 2.5) followed by loading of the sample at 4 mL min^{-1}. The analytes were desorbed from the precolumn using only the organic portion (acetonitrile) of the LC mobile phase for 1 min, and then both solvents of the mobile phase were mixed prior to entering the C-18 analytical column. Sample volumes of 2–500 mL were evaluated to ascertain the breakthrough volumes, and, as expected, the sorbents of higher surface area had greater breakthrough volumes. For the more polar analytes, the breakthrough volume appears to be slightly greater than 50 mL since the recoveries decreased at this sample volume. Recoveries of 73% for DIA and DEA were obtained at the 5 μg L^{-1} concentration when using sample volumes of 20 mL. The recoveries for atrazine and DEA were still acceptable with sample volumes of 200 mL (at 2 μg L^{-1} concentration) but the recovery for DIA decreased to 26%. In general, the *o*-carboxybenzoyl-modified PS–DVB beads (Amberchrom GC-161m) performed better than the modified PLRP-S resins with regard to recovery, but this appears to be a surface area effect. The *o*-carboxybenzoyl-modified beads and resins also have higher breakthrough volumes than their unmodified equivalents, which explains the higher retention and recovery for the polar analytes. Various chemically modified polymeric resins (e.g., acetyl, hydroxymethyl, benzoyl) and highly crosslinked sorbents for use in SPE were reviewed and discussed.[111]

On-line SPE/LC/APcI-MS was used to quantify cyanazine, cyanazine amide, and cyanazine acid in groundwater[109] with recoveries of 84–108% at the 5 μg L^{-1} concentration when using either C-18 or PLRP-S cartridges as precolumns. A Prospekt automated SPE system was used to wash the precolumn sequentially with 6 mL of acetonitrile and 4 mL of LC-grade water (pH 2.5) before loading a 20-mL water sample at a rate of 2 mL min^{-1}. The analytes were then desorbed from the precolumn into a C-18 analytical column using the mobile phase [acetonitrile–water (3 : 7, v/v) adjusted to pH 2.5 with HCl]. Detection was obtained by using atmospheric pressure chemical ionization (APcI) in both the positive and negative ion modes, and an external calibration curve was generated by analyzing 20-mL portions of pesticide-free groundwater, each fortified in the 0.01–1.5 μg L^{-1} concentration range. Significant losses of cyanazine acid occurred when the sample pH was 7 since this compound is ionic at this pH; the recoveries for this analyte were improved when the sample was adjusted to pH 2.5 prior to loading the C-18 or PLRP-S cartridges. The PLRP-S cartridge was slightly better than the C-18 cartridge at retaining cyanazine amide owing to its greater polarity.

Immunosorbents have also found applicability in on-line SPE analysis. An antibody is immobilized on to a silica support and used as an affinity ligand to retain targeted analytes. Components not recognized by the antibody are not retained and some degree of selectivity is attained.[112,113] Recoveries of 87–103% were obtained for atrazine, simazine, DEA, propazine, and terbuthylazine at the 0.2 μg L^{-1} concentration level when using immunosorbent SPE (80 mg silica and 2 mg anti-atrazine and anti-chlortoluron antibodies) on-line with LC/APcI-MS;[114] however, this method is not applicable to DIA (0% recovery). This compound may be better retained when using an

anti-simazine immunosorbent since DIA still contains the ethyl moiety in its structure. In this study, the immunosorbent was conditioned with 6 mL of a phosphate buffer solution and 3 mL of LC-grade water followed by loading 20 mL of water sample at a rate of 1 mL min^{-1}. The column was washed with 1 mL of LC-grade water before desorbing the analytes with the chromatographic mobile phase. The major ions and relative abundances of the triazines studied using APcI were detailed. Calibration curves were generated by analyzing various 20-mL portions of LC-grade water, each fortified with the desired analytes in the concentration range 0.01–0.2 μg L^{-1}. The described method was successfully subjected to an inter-laboratory validation, and the cost and time issues relating to the production of the required polyclonal antibodies were discussed. Atrazine, HA, DEA, and DIA were measured in river water and groundwater using on-line immunoaffinity extraction and reversed-phase liquid chromatography (RPLC)[115] with detection limits of 6–10 ng L^{-1} when 45-mL sample volumes were used.

In another study,[116] immunoaffinity-based solid-phase extraction (IASPE) was employed in conjunction with a PLRP-S SPE cartridge. The affinity ligand consisted of monoclonal antibodies K4E7 raised against atrazine and immobilized on beaded cellulose.[117] The IASPE cartridge (10 × 3-mm i.d.) was cleaned with 10 mL of glycine buffer and conditioned with 5 mL of LC-grade water. A sample volume of 10 mL was pumped through the cartridge followed by 5 mL of LC-grade water to remove undesired sample components. Direct desorption of the analytes into the gas chromatograph was not possible, because the packing material was not compatible with organic solvents, so on-line coupling to a PLRP-S cartridge was performed. The PLRP-S cartridge was conditioned with 2 mL of ethyl acetate and 5 mL of water at the time the IASPE cartridge was loaded with water sample. The analytes were then desorbed from the IASPE cartridge using 20 mL of glycine buffer and collected on the PLRP-S cartridge. The PLRP-S cartridge was washed with 10 mL of LC-grade water to remove the buffer and then dried for 30 min using N_2 at a flow rate of 30 mL min^{-1}. The analytes were desorbed using 100 μL of ethyl acetate at a flow rate of 70 μL min^{-1}, and this entire fraction was transferred to the gas chromatograph via a 20 cm × 110-μm i.d. metal capillary that penetrated the septum of the on-column injector. Recoveries of 64–88% were obtained for atrazine, terbuthylazine, and sebuthylazine at the 1 μg L^{-1} concentration in river water, but the recoveries were poor for simetryn, prometryn, terbutryn, and dipropetryn. This difference in recovery was likely due to the structural similarities between the acceptably recovered analytes whose chloro moiety facilitated retention on the IASPE cartridge. Recoveries of 87–101% were obtained for the thiomethyl group analytes on the PLRP-S cartridge. The important atrazine degradate DEA could not be determined owing to the large volume of glycine buffer required to desorb the other analytes from the IASPE cartridge, and the volume of wash water used for cleanup. These conditions resulted in DEA breakthrough on the PLRP-S cartridge. This was the first report of on-line coupling between immunoaffinity enrichment and GC for the determination of pesticides in water samples. One of the advantages is the high degree of selectivity for s-triazines on the IASPE cartridge; virtually no other organic compounds were retained. Therefore, no GC column deterioration was observed and matrix effects were essentially absent. The major disadvantage of the immunoassay technique is cross-reactivity to structurally similar compounds. In this work, cross-reactivity can be used to advantage to retain and enrich a selected group of compounds.

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2.1.4 Other techniques

On-line solid-phase extraction/gas chromatography/flame ionization detection (SPE/GC/FID) has also been described[118] wherein the sample preparation takes place using an HP PrepStation, and the extract, contained in a GC vial, was transferred automatically to a GC sample tray for injection. The SPE cartridge was packed with PS–DVB (PLRP-S) and conditioned with successive 10-mL portions of methanol, ethyl acetate, and LC-grade water. The cartridge was loaded with 50 mL of water sample and then washed with 5 mL of LC-grade water. The cartridge was dried for 30 min with N_2 at ambient temperature followed by elution of the analytes with 300 μL of ethyl acetate into a GC vial. The vial was transferred to the GC autosampler where 50 μL were injected for separation and analysis using an SPB5 25 m × 0.32-mm i.d., 0.25-μm film thickness, capillary column and either flame ionization or mass-selective detection. The total analysis time was 90 min. Recoveries of 91–95% were obtained for atrazine, DEA, trietazine, simetryn, terbutryn, and cyanazine at the 5 $\mu g\,L^{-1}$ concentration level in LC-grade water. River water was analyzed for triazines at the 0.6 $\mu g\,L^{-1}$ level concentration level with no practical problems.

The precolumn (or SPE cartridge) can also be used as the analytical separation column[119–121] using on-line single-short-column LC/APcI/MS/MS [ion-trap mass spectrometry (MS) or tandem quadrupole MS]. In this case, the high degree of selectivity of the tandem mass spectrometry (MS/MS) technique can be used to advantage since chromatographic resolution of targeted analytes during separation is not required, and analysis times can be significantly decreased. Trace enrichment was obtained using a 10 × 2-mm i.d. high-pressure column packed with 8-μm C-18 bonded silica.[122] Automated conditioning (2 mL of methanol and 2 mL of water) and washing (1 mL of water) of the column and loading of the sample (4 mL) were performed using a Prospekt sample-handling module (three six-port valves) and a solvent-delivery unit (SDU). After loading the sample, during which time the LC and MS instruments were in the stand-by mode, a steep LC gradient at a flow rate of 0.5 mL min^{-1} was initiated using a methanol–water mobile phase. During MS analysis, the SDU lines were flushed with methanol. The triazine peaks eluted in the range 1–7 min, and near chromatographic resolution was obtained even though using a short 10-mm column. The only peaks requiring resolution were sebutylazine and terbuthylazine, because the protonated molecular and product ions were identical. River water was fortified with the analytes at a concentration of 0.2 $\mu g\,L^{-1}$, and the results obtained were satisfactory. The linearity of the method was tested from 0.1 to 10 $\mu g\,L^{-1}$, but the lower limits could not be detected in all cases. The limit of detection (LOD) was reported as 100–200 pg [signal-to-noise ratio (S/N) = 3] injected as determined from 10-μL loop injections of standards. The LOD was 100 ng L^{-1} for atrazine, cyanazine, propazine, sebutylazine, and terbuthylazine and 200 ng L^{-1} for simazine when analyzing fortified river water samples. The total analysis time (enrichment, separation, and detection) was 20 min. The protonated molecular and product ions monitored during these experiments using ion-trap MS/MS were summarized, and the applicability of the single-column liquid chromatography/atmospheric pressure chemical ionization/ion-trap detection (LC/APcI/ITD) technique to the on-line determination of targeted triazine compounds was demonstrated.

Molecularly imprinted polymers (MIPs) have been used as sensors for the detection of triazine compounds in environmental samples.[123,124] The technique is based on the competition between labeled and unlabeled analyte for specific binding sites in the imprinted polymer. The polymer was prepared via radical polymerization of a functional monomer (e.g., diethylaminoethyl methacrylate or methacrylic acid) and cross-linker (e.g., ethylene glycol) in the presence of a template (e.g., atrazine). After removal of the template (in this example, atrazine), the polymer can be used as a three-dimensional atrazine-specific sensor system. In one study,[122] the polymer particles suspended in ethanol were incubated in the presence of 5-(4,6-dichlorotriazinyl)aminofluorescein at room temperature. The measured fluorescence of the supernatant increased in proportion to the concentration of free atrazine up to 0.01 mM owing to the release of fluorescent-labeled analyte to the solution. Conductivity has also been employed for the measurement of atrazine using MIPs in that the resistance of a solution decreased with increasing atrazine concentration.[125] Although applicable to a wide range of specific families of molecules, the technique still suffers from a relatively high noise level, low sensitivity, and interference. The MIPs are also limited by their low yield of specific binding sites, low sample load capacity, and high nonspecific binding.[126] Continued advances in the preparation of MIPs and novel approaches to detection may provide sensors with the desired selectivity and sensitivity.[127–130] The MIP technique was recently reviewed.[131]

Membrane separation coupled on-line to a flow-injection system was employed for the monitoring of propazine and terbutryn in natural water.[132] A microporous hydrophobic membrane was utilized in which the analytes were extracted from the aqueous medium into an organic solvent that was carried to the flow cell of a photodiode-array spectrophotometer. The LODs were 4–5 $\mu g\ L^{-1}$ so the technique could potentially be used for screening purposes. Samples with positive detection would require further analysis.

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Solid-phase microextraction (SPME) consists of dipping a fiber into an aqueous sample to adsorb the analytes followed by thermal desorption into the carrier stream for GC, or, if the analytes are thermally labile, they can be desorbed into the mobile phase for LC. Examples of commercially available fibers include 100-μm PDMS, 65-μm Carbowax–divinylbenzene (CW–DVB), 75-μm Carboxen–polydimethylsiloxane (CX–PDMS), and 85-μm polyacrylate, the last being more suitable for the determination of triazines.[133,134] The LODs can be as low as 0.1 $\mu g\ L^{-1}$. Since the quantity of analyte adsorbed on the fiber is based on equilibrium rather than extraction, procedural recovery cannot be assessed on the basis of percentage extraction. The robustness and sensitivity of the technique were demonstrated in an inter-laboratory validation study for several parent triazines and DEA and DIA.[135] A 65-μm CW–DVB fiber was employed for analyte adsorption followed by desorption into the injection port (split/splitless) of a gas chromatograph. The sample was adjusted to neutral pH, and sodium chloride was added to obtain a concentration of 0.3 $g\ L^{-1}$. During continuous stirring, the fiber was dipped into the sample for 30 min at room temperature. Subsequently the analytes were desorbed into the gas chromatograph for 5 min and analyzed using either nitrogen–phosphorus detection (NPD) or ion-trap detection (ITD). The average LODs were in the range 4–24 $ng\ L^{-1}$ for the parent compounds and 20 and 40 $ng\ L^{-1}$ for DIA and DEA, respectively. The study was considered valid for all the analytes except DIA; only one of the 10 participating laboratories reported results for

this degradate. The advantages of SPME include little modification to existing GC and LC hardware, faster sample preparation, and solvent-free analysis.[136]

Supercritical fluid extraction (SFE) is generally used for the extraction of selected analytes from solid sample matrices, but applications have been reported for aqueous samples. In one study, recoveries of 87–100% were obtained for simazine, propazine, and trietazine at the 0.05 $\mu g\,mL^{-1}$ concentration level using methanol-modified CO_2 (10%, v/v) to extract the analytes, previously preconcentrated on a C-18 Empore extraction disk.[137] The analysis was performed using LC/UV detection. Freeze-dried water samples were subjected to SFE for atrazine and simazine,[138] and the optimum recoveries were obtained using the mildest conditions studied (50 °C, 20 MPa, and 30 mL of CO_2). In some cases when using LLE and LC analysis, co-extracted humic substances created interference for the more polar metabolites when compared with SFE for the preparation of the same water sample.[139]

2.1.5 Sample storage

Sample storage is receiving increased attention owing to stability issues created by potential chemical and biochemical mediated transformations of analytes during the storage time interval. Typically, water samples are collected in amber-glass bottles and shipped chilled to the analytical laboratory where they are stored at 4 °C until analyzed. In some cases, loss for some analytes was observed after only 14 days of storage.[140] Although analyte transformation during sample storage is a serious concern for many pesticides, some of the triazine compounds and their degradates are stable in surface water, groundwater, or deionized water for as long as 2 years[141] when stored in the dark at 4 °C. They also appear to be stable for up to 14 months when stored in the dark at room temperature,[142] and the addition of special biological inhibitors was not required. These studies included atrazine, simazine, DIA, DEA, DACT, ametryn, and prometryn. Nevertheless, analysis as soon as possible after sample collection is generally preferred. Studies using C-18 and GCB[143] (34 selected pesticides at the 5–15 $\mu g\,L^{-1}$ concentration level) and PLRP-S SPE cartridges[144] (17 selected pesticides at the 10 $\mu g\,L^{-1}$ concentration level) have demonstrated the stability of these selected pesticides when stored in the cartridges under various conditions. Analytes were stable for 21 days on the C-18 and GCB cartridges and for 7 weeks on the PLRP-S cartridge. A storage temperature of −18 °C seemed to improve analyte stability compared with storage at 4 °C , but there appeared to be little difference in the stability when comparing storage temperatures of 4 °C and room temperature. The sample pH had a significant effect on the stability of those analytes with acidic or basic properties. The presence of water in the stored SPE cartridge appeared to have little effect on the stability of the analytes. Advantages of performing the extraction as soon as possible include the elimination of potential analyte loss and savings in sample storage space. In another study,[145] analytes stored on PS–DVB cartridges for 3 months at −20 °C showed excellent stability. The cartridges, after appropriate conditioning and washing, were loaded with 50-mL water samples containing 5 $\mu g\,L^{-1}$ of atrazine, simazine, DEA, DIA, and cyanazine (and also several other nontriazine pesticides) and stored for various periods of time up to 3 months. Three storage temperatures (−20 and 4 °C and room temperature) were studied. At pre-selected time intervals, some of the cartridges were thawed for 4–6 h at room temperature and subjected to analysis

using on-line SPE LC/DAD or LC/APcI/MS. The results indicated that storage at −20 °C for 3 months was best, but for most compounds storage at 4 °C was also acceptable. Storage at room temperature was not recommended, but this was because of loss of carbamate rather than triazine compounds. The advantage of performing an immediate extraction of the sample using SPE is the potential to perform on-site monitoring.[146]

3 Analytical methodology for soil samples

The quantitative extraction of triazines from soil is more complex than the isolation of these compounds from water. The binding mechanism of triazines and soil humic acids is not well understood. Proton transfer may be favored for humic acids of high acidic functional group content and for s-triazines of low basicity, and electron-transfer mechanisms may be favored for humic acids of low acidic functional group content and for s-triazines of high basicity. The specific molecular structure appears to affect the binding interactions of s-triazines with humic acids more than the overall s-triazine class structure.[147] Experiments with radiolabeled triazines show that in soils with 'aged' residues, a fraction of the compound becomes nonextractable, at least to the more commonly employed extraction techniques, and this 'bound' portion requires more exhaustive and/or rigorous extraction conditions to extract the residue quantitatively if extractable at all. Thus, the use of laboratory-fortified samples for procedural recovery evaluation becomes questionable. Various soil characteristics (percent sand/silt/clay, pH, cation-exchange capacity, etc.) may affect the extractability of triazine residues, but the total organic carbon (TOC) content of the soil, primarily humic acids, is considered the main source of absorption. A method using SPME was reported for the determination of the adsorption coefficients of triazines in soil.[148]

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In contrast to the high accuracy and precision level of modern chromatographic instrumentation, the extraction and recovery of trace organic analytes from solid sample matrices such as soil represent the slowest and most error-prone aspects of an analytical method. Prior to analysis, the analyte(s) must be extracted from the soil followed by some kind of enrichment and/or sample purification and concentration step(s). Historically, the most common technique employed for the extraction of triazine compounds from soil is LSE, e.g., Soxhlet, mechanical shaking, or stirring. The extract is then typically subjected to LLE and/or cleanup using Florisil, alumina, or silica gel column chromatography to enrich and concentrate the analyte(s) prior to analysis. Although LSE is still the most frequently used technique, there are reports devoted to applications of sonication and SFE and also a few reports addressing the applications of microwave and sub-critical water extraction. Further, the popularity of SPE for extract purification has increased significantly and has almost entirely replaced the use of column chromatography. This is because the cartridges are relatively inexpensive on a per sample basis, and there is a need to reduce the use and disposal of organic solvents for economic and environmental reasons. In addition, the use of SPE for sample preparation can be automated. The technique is still dependent on first removing the analytes of interest from the soil matrix via other extraction techniques, and most methods require the use of at least one sample purification procedure after extraction and prior to injection on analytical instrumentation.

3.1 Liquid–solid extraction

In Soxhlet extraction, soil samples are typically dried and then sieved to the desired particle size prior to transferring the sample to a thimble to be inserted into the Soxhlet extractor. Solvents such as methanol are then distilled, condensed, and allowed to percolate through the soil in the thimble for some pre-determined extraction time, typically varying from 2 to 24 h, to enrich the condensed solvent at the bottom of the apparatus with analyte(s). Atrazine, simazine, cyanazine, DEA, and DIA at the 1 $\mu g\,g^{-1}$ concentration level were quantitatively recovered from soil using Soxhlet extraction and Florisil column or gel permeation chromatography (GPC) cleanup prior to analysis using GC/MS and LC/TSP-MS.[149] The soil was freeze-dried and sieved through a 120-μm sieve prior to extraction with methanol–water (9 : 1, v/v) for 12 h. In another study, 150 mL of methanol–water (2 : 1, v/v) were used as the Soxhlet extraction solvent followed by C-18 SPE and GC/MS analysis. The recoveries for atrazine, simazine, propazine, terbuthylazine, desmetryn, ametryn, and terbutryn were quantitative but poor for DEA (likely due to loss during C-18 SPE) at the 5–10 $\mu g\,kg^{-1}$ concentration level.[150] The soil used in these experiments was air-dried and sieved to 2 mm prior to extraction. A recent study reported the use of automated hot solvent extraction (Buchi Extraction System allowing the simultaneous extraction of four samples) for the extraction of atrazine, DEA, and DIA from soil.[151] Air-dried and sieved (2.0-mm) soil samples (20 g) in glass thimbles were extracted for 30 min with 120 mL of boiling dichloromethane–acetone (13 : 7 or 3 : 1, v/v). The extract was subjected to GPC cleanup and LC/UV analysis. The recoveries were 81–98% when the soil TOC content was $\leq$2.5% but decreased as the TOC increased, especially for DEA and DIA, indicating a decrease in extraction efficiency for the more polar analytes as the TOC content of the soil increases. A method for the determination of triazines and other pesticides in marine sediment samples was reported using Soxhlet extraction, SPE, and GPC.[152] An 80-g sample was extracted for 6 h with acetone prior to solvent evaporation and purification using a C-18 SPE cartridge. Further purification and isolation was achieved using high-performance gel permeatron chromatography/ultraviolet (GPC/UV) to separate the analytes from the high molecular weight humic acids and elemental sulfur, and the final analysis was accomplished using gas chromatography/alkali flame ionization detection (GC/AFID). However, the recoveries were <70% for atrazine, simazine, atraton, propazine, prometryn, and terbutryn and were only 12% for DEA and desethylterbutylazine. The recovery was 74% for terbuthylazine.

Mechanical shaking or stirring of the soil sample with an extraction solvent is another frequently used form of LSE. Mechanical rotary shaking was employed to extract quantitatively cyromazine and its degradate melamine at the 10 $\mu g\,kg^{-1}$ concentration level from 20 g of soil using acetonitrile–0.05 M ammonium carbonate (7 : 3, v/v) for 30 min.[153] Additional purification of the sample extract was obtained using an SCX resin, and the sample was analyzed using GC/MS or LC/UV. Recoveries of 87–97% were obtained for atrazine, HA, DEA, and DIA when extracting 50 g of soil with 150 mL of methanol on a rotary shaker[154] and quantifying by thin-layer chromatography and densitometry. Atrazine was quantitatively recovered by stirring 25 g of soil with 100 mL of dichloromethane for 2 h.[155,156] The soil was air-dried

and sieved to 55-mesh prior to extraction. Quantitative recovery for prometryn was obtained when 60 g of soil were shaken with 140 mL of methanol–water (4 : 1, v/v) for 1 h[157] followed by phenyl-SPE and GC/NPD analysis. In another study,[158] the soil was dried and sieved to pass 2 mm before 50-g portions were shaken for 15 min with 50 mL of 0.01 M NaOH and subjected to centrifugation for 10 min at 4000 rpm. The extraction was performed twice, and the two fractions were combined prior to the addition of 10 mL of 1.0 N HCl. The pooled supernatant was partitioned three times with 50-mL portions of dichloromethane, and the combined organic fraction was dried through anhydrous sodium sulfate and concentrated prior to analysis using LC/UV detection. The method was designed to capture compounds from several different compound classes, but recovery values for terbuthylazine and propazine were not presented. Quantitative recoveries were obtained for atrazine, DEA, DIA, DACT, and HA when 10 g of sediment sample were shaken for 30 min at 300 rpm with 25 mL of methanol–0.1 N HCl (1 : 1, v/v).[159] The SPE cartridges employed were in-tandem C-18 and SCX. Quantitative recoveries were obtained for atrazine, DEA, and DIA on the C-18 cartridge, but the SCX SPE cartridge was required for the quantitative recovery of DACT and HA. Several other SPE cartridges, C-8, C-2, CH, CN, and 2OH, were evaluated with varying results. Final analysis was accomplished using liquid chromatography/photodiode array (LC/PDA). Mechanical shaking in combination with elevated temperature has also been used for the extraction of triazines from soil.[160] A 20-g soil sample was equilibrated for 1 h with 5 mL of water on a mechanical shaker. Methanol (15 mL) was added to form a slurry, and the sample was heated at 75 °C for 30 min with periodic vortex mixing. The sample was shaken for an additional 15 min to allow cooling and subjected to centrifugation. The clear supernatant was decanted, and the procedure was repeated. The pooled extracts were purified using a C-18 SPE cartridge and ethyl acetate as elution solvent followed by further purification using an SAX SPE cartridge to remove colored humic acids. Final analysis was accomplished using GC/MS, and the recoveries were 75% for atrazine, simazine, propazine, DEA, and DIA. Interestingly, DEA and DIA were not recovered when using Soxhlet extraction. All traces of methanol had to be removed from the soil extract to avoid analyte loss during the C-18 SPE step since even as little as 1% methanol in the SPE load fraction adversely affected the recoveries for the dealkylated degradates. Many different solvents and combinations of solvents for 'shaking extractions' of soil have been studied and reported over the last four decades.[161–168] Overall, methanol appears to be the most often employed organic solvent for the extraction of triazines from soil, and solvent mixtures containing water appear to improve the extractability of the more polar or hydrophilic analytes.[169,170]

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3.2 Sonication

Ultrasonication was reported for the extraction of triazines from soil, previously sieved to 2 mm and stored at −18 °C, prior to analysis using GC/NPD and GC/ITD.[171] A 5-g soil sample was placed in a polypropylene column and extracted for 15 min with 4 mL of ethyl acetate in an ultrasonic bath at room temperature. Subsequently, the solvent was filtered and collected in a graduated tube, and the extraction was repeated for another 15-min period using a second 4-mL portion of ethyl acetate. The two extracts

were pooled and evaporated to a volume of 2–5 mL for analysis. The recoveries for atrazine, terbuthylazine, prometryn, terbutryn, and cyanazine were quantitative at the 0.2 $\mu g\,kg^{-1}$ concentration level. The procedure was optimized not only for triazines but also for other nitrogen-containing compounds. The more polar degradates were not studied. Sonication and C-8 SPE disks were used for the extraction of atrazine from soil.[172] A 5-g soil sample was subjected to sonication in 5 mL of distilled water for 15 min followed by sonication for 15 min in 5 mL of acetone. The water–acetone–soil suspension was filtered and purified through a C-8 disk prior to analysis using GC/NPD. The recovery for atrazine was 71% at the 0.03 $\mu g\,g^{-1}$ concentration level. In another study, 7 g of soil were sonicated for 30 min with 15 mL of acetone. The clear supernatant obtained after centrifugation was evaporated to dryness and reconstituted to 3.5 mL in acetone prior to analysis using 100-μm PDMS or polyacrylate (PA) SPME fibers and direct injection GC/MS.[173] Owing to the nature of the SPME process, the recoveries could not be evaluated in the conventional manner. However, the PA fiber appeared to be better for the determination of the more polar triazines such as DEA, DIA, and DET, but the PDMS fiber was better for the parent compounds, atrazine, simazine, terbuthylazine, and cyanazine.

3.3 Microwave extraction

Microwave-assisted solvent extraction (MASE) is still relatively new and only a few applications have been reported for soil analyses.[174,175] The technique is based on the absorption of microwave energy to raise the temperature and pressure of the sample and the associated bulk and interstitial solvent to induce increased diffusion of the analyte(s) from the sample matrix into the surrounding solvent. Reductions in solvent usage, operational simplicity, and speed of extraction are noted as advantages. The effects of various microwave operating parameters on the extraction efficiency of atrazine, simazine, DEA, and DIA from various soil types were evaluated.[176] These authors concluded that the optimum operating parameters were not very critical as far as their effect on extraction efficiency was concerned. However, one disadvantage was the lack of discrimination between the extraction of the analyte(s) and other potentially interfering sample components. In a related study,[177] the extracts from soil samples subjected to microwave extraction were analyzed for atrazine, simazine, DEA, and DIA at the 2 $\mu g\,kg^{-1}$ concentration level using GC/NPD and GC/MS without further cleanup. This worked well for soil containing $<5\%$ TOC, but the direct injection of extracts from soil containing $>5\%$ TOC reduced the NPD response and shortened the life of the capillary column. Soil samples containing 5–30% TOC were successfully extracted and analyzed for these triazines when further extract cleanup was performed using 100-mg silica SPE cartridges.

3.4 Supercritical fluid extraction

Numerous applications of SFE were published during the 1980s soon after the availability of commercial instrumentation. Supercritical fluids (SFs) have useful characteristics for the extraction of trace analytes from solid samples, most notably

solvent strengths that approach those of liquids and viscosities and diffusivities that approach those of gases. These solvation power and improved mass transfer advantages make SFE a potentially viable technique for the extraction of triazines from soil. Although several SFs have been employed in the practice of SFE (e.g., ammonia, pentane, N_2O, SF_6, etc.), CO_2 continues to be used most often owing to its low critical temperature (31.3 °C), moderate critical pressure (1070 psi), nontoxic nature, low cost, and solubility in many organic solvents. The addition of modifiers such as methanol, ethanol, etc., to the SF provides some selectivity to the extraction process and enhances the ability of the technique for the extraction of polar analytes. The solubilities of atrazine, simazine, ametryn, and prometryn in SF CO_2 were studied[178] and, as expected, the solubility increased with increasing pressure. This was due to the decreased mean intermolecular distance of the CO_2 molecules that increased the interaction between the solute and solvent molecules. The solvent density decreases rapidly with small increases in temperature, but at higher pressures, the solvent density is only slightly affected by temperature. The solubilities of atrazine and simazine (the −Cl adding more polarity than the $-SCH_3$ group) are lower than those for ametryn and prometryn at the same pressure. Pressure and the amount of modifier used with respect to cell volume appear to be two of the more important parameters affecting extraction efficiency.

Atrazine and HA at the 20 mg kg^{-1} concentration level were quantitatively extracted from soil using CO_2 as the SF and methanol (10%, v/v) as modifier.[179] The addition of water as a modifier added little to the recovery. The optimum conditions were 60 min, 65 °C, and 300 bar. Under all conditions studied, the recoveries were poor for deisopropyldesethyl-2-hydroxyatrazine (the hydroxy version of DACT). Final analysis was performed using LC/UV detection and few peaks were detected other than the analytes, indicating some selectivity during the extraction. These authors later reported a comparison study of SFE versus LSE[180] for the extraction of atrazine, HA, OH-DACT, and DEA. Interestingly, the recovery of atrazine was slightly higher when using the LSE technique, but the polar degradates could not be quantified at all owing to UV-absorbing interference. In this study, the LSE procedure consisted of vigorous stirring of the soil sample for 4 h in 100 mL of methanol–water. The time required was 45 min per sample using SFE. Atrazine, DEA, HA, terbuthylazine, deethylterbuthylazine, and OH-terbuthylazine were extracted from soil at the 5 μg g^{-1} concentration level using three procedures, SFE with methanol-modified CO_2, Soxhlet extraction with methanol, and shaking with acetone–water.[181] Final analysis was performed using LC/PDA. The recoveries were quantitative and comparable for all three techniques for the chlorinated triazines including the dealkylated degradates. However, the recoveries for both the hydroxy degradates using SFE (4% for HA and 21% for OH-terbuthylazine) were much lower than the 50% recoveries obtained using the more classical extraction procedures. Other investigations in which SF CO_2 with methanol as modifier was used to extract triazines from soil showed that higher recoveries could be obtained for parent triazines when the soil moisture content was 10–20%.[182] In this study, the recoveries obtained using SFE were comparable to those obtained using Soxhlet extraction with methanol. In another study, the extraction efficiencies for atrazine, terbuthylazine, and propazine from soil using SFE (CO_2 and acetone as modifier), ultrasonication in water (and methanol or water–methanol mixtures), hot extraction (boiling the soil in water), and Soxhlet extraction (in methanol)

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were compared.[183] The authors concluded that none of these procedures provided quantitative recoveries for all three analytes. Based on the recovery data and study design, they chose ultrasonication as the extraction technique for further study. This technique was rapid, multiple samples could be prepared in parallel, and the recoveries were equal to those of the other three techniques for these three compounds. Supercritical CO_2 and various compositions of binary (CO_2 and methanol) and ternary mixtures (CO_2, methanol, and water) were studied for the extraction of atrazine, HA, DEHA, DACT, DIA, and DEA from sediment samples and compared with the results obtained using Soxhlet extraction with methanol.[184] The recoveries were almost always improved using SFE versus Soxhlet extraction, but the optimum SFE parameters were different for each analyte. The authors demonstrated that there was no advantage in increasing the temperature higher than 50 °C or pressure higher than 306 atm. There was also no advantage in adjusting the pH prior to extraction since the altered cationic or anionic form (depending on pH) still had available + or − adsorption sites in the sediment matrix. The addition of $CaCl_2$ as a modifier improved the recoveries for all analytes, presumably owing to the competition of Ca^{2+} ions and analyte for the available adsorption sites. The recoveries were 67–78% for atrazine, 103% for HA, 88% for DEHA, 72–73% for DACT, 78% for DIA, and 68–69% for DEA using SFE.

3.5 Subcritical fluid extraction

Subcritical water has potentially useful characteristics for the extraction of triazines from soil. Subcritical water is low cost, readily obtainable, easily disposed, and nontoxic, and its solubility characteristics can be varied as a function of temperature as long as the water is compressed to >40 bar to maintain the liquid state (below the critical temperature and pressure). Subcritical water was used for soil remediation purposes to extract terbuthylazine, its three dealkylated degradates, and three of its hydroxy degradates from soil.[185] The analytes were eluted from a 3-g soil sample/2-g sand mixture using 10 mL of phosphate-buffered water at 100 °C for about 9 min and collected on a GCB SPE cartridge. The cartridge was inverted and eluted with 1.5 mL of methanol and 6 mL of dichloromethane–methanol (4 : 1, v/v, containing 5 mmol HCl). After removal of the eluate, the residues were reconstituted in 150 μL of water–methanol (2 : 3, v/v) and acidified with HCl to pH 3 before final analysis using LC/MS/MS. The recoveries were 95–103% at the 30 $\mu g\,kg^{-1}$ concentration level. A comparison of this procedure to the results obtained analyzing the same 'aged' soil using Soxhlet extraction (methanol) and a room temperature batch extraction (phosphate buffer–acetonitrile) showed that the subcritical water extraction procedure consistently recovered higher quantities of the analytes. The solubilities of atrazine, simazine, and cyanazine in subcritical water and modified water (containing ethanol or urea) were reported at temperatures ranging from 50 to 125 °C and 50 atm.[186] Adding co-solvent and increasing the temperature increased the solubilities of these three triazines, and the analytes did not thermally degrade or hydrolyze at the upper temperatures used in this study. In pure water, the solubilities increased 3-fold for each 25 °C increase in temperature.

Soil leachates were analyzed for ametryn, prometryn, and terbuthylazine using 85-μm polyacrylate and 100-μm PDMS SPME fibers.[187] The results obtained

using SPME correlated well with the concentrations of these compounds obtained by solvent extraction in methanol and analysis using LC/UV detection. Soil leachates were also analyzed using C-18 SPE disks for atrazine, simazine, and propazine with quantitative recovery.[188]

3.6 On-line SFE

On-line supercritical fluid extraction/gas chromatography (SFE/GC), supercritical fluid extraction/supercritical fluid chromatography (SFE/SFC),[189–191] supercritical fluid extraction/liquid chromatography (SFE/LC),[192,193] and supercritical fluid extraction/capillary electrophoresis (SFE/CE)[194] applications were reported, and one or more of these techniques may eventually become useful approaches to screening large numbers of samples. The technology is still rather complex and not easily amendable to routine use. Overall, realistic advantages of using SFE versus other extraction techniques have not been demonstrated. Whether or not this technique develops into one of widespread use remains to be determined.

4 Analytical methodology for crops, food, feed, and animal tissues

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Procedures utilized for the extraction of triazine compounds from crops, food, feed, and animal tissues are still dominated by sample homogenization in polar organic solvents such as methanol and acetonitrile (in combination with water), dichloromethane, or acetone and acetone–water combinations using a high-speed blender or Polytron apparatus. As with soil, methanol appears to be the most often used solvent for these applications. After filtering the initial extract, portions are typically subjected to purification using LLE, SPE, SFE, or other steps in combination prior to the final analysis. Column chromatography sample preparation using bulk quantities of silica, alumina, Florisil, etc., while still occasionally employed, has generally been replaced with SPE.

Quantitative recoveries for atrazine, DEA, simetryn, and secbumeton at the $0.10\,mg\,kg^{-1}$ concentration level in crop samples (e.g., apples, cherries, corn, oranges, plums, etc.) were obtained using a combination of blending, LLE, SFE, and final analysis using GC/NPD (confirmatory analyses using GC/MS).[195,196] Crop samples (100 g) were blended with methanol and filtered. A portion of the filtrate equivalent to 50 g of crop was diluted with water and saturated salt solution and partitioned twice with dichloromethane. This fraction was dried and solvent-switched to hexane prior to additional cleanup using SCX SPE. The method is applicable to the determination of 19 triazines and four of their degradates. Quantitative recoveries were obtained for grass samples (35 g) fortified at the $0.14\,mg\,kg^{-1}$ concentration level with atrazine, simazine, terbuthylazine, demeton, and cyanazine after extraction by homogenization in a blender with 100 mL of acetone.[197] The analytes were extracted via LLE into dichloromethane and subjected to further purification using GPC and final analysis using GC/MS. Matrix-matched standards were required to improve the accuracy of the method.

Ultrasonication was employed to extract atrazine and simazine from watermelon (this method is also applicable to soil analysis) by freeze-drying, crushing, and sieving the crop to 120 μm.[198] A 100-g watermelon sample was ultrasonically extracted with 50 mL of methanol for 5 min and filtered. This step was repeated a total of three times, and the fractions were combined before drying the pooled fraction through a column of anhydrous sodium sulfate. The column was washed with 50 mL of dichloromethane, and the dichloromethane wash was collected with the dried methanol fraction. The combined fractions were evaporated to dryness, and the dry residue was reconstituted in 2 mL of benzene. A derivatization reagent, 4-(2-phthalimidyl)benzoyl chloride, was added to the final fraction, which was shaken for 20 min in a 20 °C water-bath followed by centrifugation at 4000 rpm. The supernatant was analyzed using LC/UV detection at 345 nm since the derivatized forms of atrazine and simazine were reported to have much higher molar absorption coefficients than the underivatized forms. Recoveries of 90–95% were obtained at the 0.03 mg kg^{-1} concentration level.

Quantitative recoveries of atrazine, simazine, propazine, and terbuthylazine (>80%) and near quantitative recoveries of cyanazine, simetryn, and prometon (65–72%) were obtained with apple, carrot, celery, corn, potato, and pea extracts fortified at the 0.01 mg kg^{-1} concentration level using immunoaffinity chromatography.[199] Crop samples of 5 g were extracted in 20 mL of methanol using Polytron homogenization. The extract was centrifuged, and a 5-mL portion of the supernatant (equivalent to 1 g of tissue) was evaporated to 0.2–0.3 mL at 50 °C under a gentle stream of nitrogen. This fraction was diluted to 8 mL with aqueous phosphate buffer solution for further purification using a 500-mg SAX SPE cartridge. The analytes were eluted with methanol–water (3 : 1, v/v), and the eluate was evaporated to 1 mL prior to dilution to 5 mL with phosphate buffer. This fraction, after appropriate conditioning of the column, was loaded on to an atrazine immunoaffinity cartridge, and the analytes were eluted with methanol–water (7 : 3, v/v). After evaporation of the eluate to 0.5 mL, a 100-μL aliquot was analyzed using LC/UV detection. One of the primary advantages of this method was that methanol (and in much smaller quantities) was the only organic solvent used other than the acetonitrile employed for the LC mobile phase. An economic advantage was that the immunoaffinity cartridge could be re-used at least 30 times without carryover problems. The primary disadvantage of using immunoaffinity columns is the long and arduous process required (as long as 12 months) for the development of a selective antibody for each individual analyte.

In another study, catfish samples were homogenized in ethyl acetate, and the residues were partitioned into acetonitrile and petroleum ether, subjected to C-18 SPE purification, and analyzed using LC/UV detection.[200] Quantitative recoveries were obtained for atrazine, simazine, and propazine in the 12.5–100 μg kg^{-1} concentration range.

Beef kidney samples were analyzed for atrazine by dispersing 0.5-g portions of kidney with 2-g portions of XAD-7 HP resin for matrix solid-phase dispersion.[201] By using a mortar and pestle, a powder-like mixture was prepared that was subjected to subcritical extraction using ethanol-modified water at 100 °C and 50 atm. The ethanol–water extract was sampled using a CW–DVB SPME fiber for direct analysis using ion-trap GC/MS, and the recoveries were quantitative for atrazine at the 0.2 mg kg^{-1} fortification level.

Whole eggs were extracted and analyzed for 10 parent triazine compounds at the 0.1 mg kg^{-1} concentration level using SFE with unmodified CO_2, off-line collection and purification using a Florisil SPE cartridge, and analysis using GC/NPD.[202] The SFE conditions were 680 atm and 50 °C, and the recoveries were quantitative for the 10 parent compounds. This method was compared with a solvent extraction method for determining atrazine, DEA, and DIA concentrations in 'real' samples, and the SFE method detected consistently higher concentrations of these three compounds. Most of the SFE methods reported previously required the use of modified CO_2 to extract some of the more polar degradates. The authors concluded that the lipids in the eggs may have acted as co-solvents, and that SFE at 680 atm (10 000 psi) increased the polarity of the SF sufficiently to extract analytes as polar as DEA and DIA.

A method for the analysis of wine for simazine (and other nontriazine compounds) was reported that required the LLE of 200 mL of wine three times with dichloromethane followed by column chromatography using 15 g of silica gel or C-18 SPE for comparison purposes.[203] Final analysis was accomplished using GC/NPD. Recoveries were good at the 0.25 mg kg^{-1} concentration level when using either the bulk silica gel or the SPE cartridge. However, the final extracts from the SPE procedure were pale in color and contained a few interfering peaks. Atrazine, simazine, terbuthylazine, DEA, DIA, and deethylterbuthylazine were determined in wort and commercial beer using LC/UV detection and confirmation using LC/PDA and GC/MS.[204] The initial isolation of the analytes was performed using LLE (Extrelut column) or PS–DVB SPE followed by further purification using SCX and C-18 SPE cartridges. The recoveries ranged from 63 to 82% with little difference obtained when the initial extraction was performed using either PS–DVB SPE or the Extrelut column as determined from liquid scintillation counting measurements of ^{14}C-labeled analytes in the extract. Overall recoveries were lower for wort than for beer, presumably owing to the more complex nature of the sample. The use of the Extrelut column helped avoid emulsion issues that frequently arise using LLE for the extraction of liquid foods such as milk, wort, and beer. The claimed detection limits ranged from 0.1 to 0.75 μg L^{-1}.

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5 Analytical methodology for biological fluids

Applicators, mixers, loaders, and others who mix, spray, or apply pesticides to crops face potential dermal and/or inhalation exposure when handling bulk quantities of the formulated active ingredients. Although the exposure periods are short and occur only a few times annually, an estimate of this exposure can be obtained by quantifying the excreted polar urinary metabolites. Atrazine is the most studied triazine for potential human exposure purposes, and, therefore, most of the reported methods address the determination of atrazine or atrazine and its metabolites in urine. To a lesser extent, methods are also reported for the analysis of atrazine in blood plasma and serum.

Urine was analyzed for atrazine, DEA, DIA, and DACT at the 0.1–100 μg kg^{-1} concentration range but detailed recovery information was not provided.[205] A 5-mL urine sample was mixed in a tube for 15 min with 5 mL of diethyl ether and 0.7 g of sodium chloride. After separation of the layers, the aqueous fraction was mixed with 5 mL of ethyl acetate for a second partitioning step. The two organic fractions

were pooled and evaporated to dryness prior to reconstitution in 100 μL of acetone for analysis using GC/NPD. Quantitative recoveries of 71–118% were obtained for atrazine, DEA, and DIA in urine at the 0.01 mg kg^{-1} concentration level when the final fractions were analyzed using GC/MS.[206] A 10-mL urine sample was adjusted to pH 10, and 1 g of sodium sulfate was added prior to filtering and passage of the mixture through a C-2 SPE cartridge. The column was dried for 10–15 min, and the analytes were eluted with 2 mL of ethyl acetate. This provided a final fraction suitable for analysis using either GC/NPD or GC/MS. Mean recoveries of 115, 113, 112, and 97% were obtained for atrazine, DEA, and DIA, and DACT, respectively, when analyzing urine samples fortified at the 1–200 μg kg^{-1} concentration range using GC/MS in the selected ion monitoring (SIM) mode.[207] This validated method also passed an independent laboratory validation (ILV) study (ruggedness test). A 25-mL portion of urine was mixed with acetonitrile and Celite 545 to precipitate proteins. The quantity of acetonitrile in this fraction was reduced via rotary evaporation before acidification, 5 mL of methanol were added, and further purification was conducted using SAX and silica SPE cartridges. The ethyl acetate eluent from the silica SPE cartridge was evaporated to dryness, and the dry residues were reconstituted in acetone for analysis. Recoveries of 106, 104, 107, and 95% were obtained for atrazine, DEA, DIA, and DACT, respectively, when using this method to analyze urine samples during a worker exposure study.

Eight parent triazine compounds were determined in human serum and urine at the 0.5 mg kg^{-1} concentration level using C-18 SPE cartridges for extraction and purification purposes and GC/NPD[208] for detection and quantitation. The serum and urine recoveries were reported to be >65 and >97%, respectively, but detailed recovery data were not presented. At these high fortification levels, the chromatograms were relatively free of interfering peaks. A method was reported for the determination of atrazine in human blood plasma for clinical cases involving ingestion/poisoning.[209] A plasma sample volume of 2 mL was mixed and shaken for 5 min with 6 mL of dichloromethane followed by centrifugation for 5 min at 4000 rpm. The two phases were separated, and the aqueous fraction was partitioned a second time with another 6-mL portion of dichloromethane. The two organic phases were pooled and evaporated to dryness prior to reconstitution in 50 μL of water–methanol (2 : 3, v/v) for analysis using LC/UV detection. Recoveries of 72 and 88% were reported for atrazine at the 6.25 and 100 μg L^{-1} fortification levels, respectively.

6 Analytical methodology for air samples

Pesticides may enter the atmosphere during spray applications of the formulated product, by volatilization, through management practices, via wind-distributed soil particles containing absorbed pesticides, etc. Several analytical methods have been reported over the last 30 years for the determination of pesticides in air, and all involve the passage of known volumes of air for a pre-defined time period through an adsorbent material to trap the desired analytes. These analytes are then extracted, concentrated, and analyzed. A few analytical methods have been reported for the determination of triazine compounds in air in the last decade.

Polyurethane foam (PUF) plugs were used to trap atrazine, simazine, DEA, and DIA when air was drawn through experimental chambers at 2.9 m^3 min^{-1}.[210] The plugs

were subjected to Soxhlet extraction for 3 h with 150 mL of ethyl acetate, followed by evaporation of the solvent and analysis using GC/NPD. Gaseous and particulate-associated atrazine and 12 other compounds were monitored by pumping air through a 30-cm glass-fiber filter and a cartridge containing 20 g of XAD-2 resin for 24 h at a rate of 10–15 $m^3\ h^{-1}$ using a high-volume sampler.[211] The filter and XAD resin cartridge were subjected to Soxhlet extraction for 12 h in hexane–diethyl ether followed by evaporation of the solvent to 1 mL. This concentrated extract was separated into three fractions (atrazine is in the third fraction) using LC, and the fractions were each manually collected prior to final analysis using GC/ECD. Recovery data were not presented since the sampling mechanisms were difficult to reproduce under laboratory conditions. The applicability of the method was demonstrated by analyzing samples collected in the field. This multi-residue method was later expanded to include the use of GC/ITD for analysis.[212]

7 Instrumentation

All previous discussion has focused on sample preparation, i.e., removal of the targeted analyte(s) from the sample matrix, isolation of the analyte(s) from other co-extracted, undesirable sample components, and transfer of the analytes into a solvent suitable for final analysis. Over the years, numerous types of analytical instruments have been employed for this final analysis step as noted in the preceding text and Tables 3 and 4. Overall, GC and LC are the most often used analytical techniques, and modern GC and LC instrumentation coupled with mass spectrometry (MS) and tandem mass spectrometry (MS/MS) detection systems are currently the analytical techniques of choice. Methods relying on spectrophotometric detection and thin-layer chromatography (TLC) are now rarely employed, except perhaps for qualitative purposes.

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7.1 Gas chromatography

Nitrogen/phosphorus detection (NPD), electron-capture detection (ECD), flame photometric detection (FPD), and flame ionization detection (FID) have been widely employed in GC analysis for several decades. Some selectivity for the nitrogen-containing triazines is obtained using NPD, and ECD is particularly sensitive to halogenated compounds. The nonselective FID is rarely used for triazine-related analyses, but FPD in its sulfur mode is particularly useful for the detection of the methylthiotriazines. Since these detection methods are still often used in today's laboratories, one must exercise caution and not rely solely on the use of retention time for identification purposes, especially for analytes at the sub-$\mu g\ kg^{-1}$ concentration levels. Positive detection in samples analyzed using non-MS screening procedures should be reanalyzed for confirmatory purposes utilizing an MS-based method. While useful, reanalysis of the sample using a column with an alternative stationary phase is still not as reliable as MS for confirmation of the analyte's identity.

Chromatographic systems were finally coupled with relatively inexpensive, yet powerful, detection systems with the advent of the quadrupole mass selective detector (MSD). The operational complexity of this type of instrumentation has significantly declined over the last 15 years, thus allowing routine laboratory use. These instruments

using electron ionization (EI) and operated in the SIM mode offer sensitivity comparable to, if not better than, that of earlier detectors but with the added benefit of obtaining confirmatory information via the monitoring of selected qualifier ions. Further, electron ionization/mass spectrometry/selected ion monitoring (EI/MS/SIM) is less affected by sample components that typically interfere during analyses using NPD, ECD, or FPD. Owing to insufficient sensitivity, operation of an MSD in the full-scan mode (acquisition of the total EI mass spectrum) is not typically performed during the analysis of environmental samples containing sub-$\mu g\,kg^{-1}$ concentrations of analytes. Chemical ionization (CI) in the positive and negative ion mode is sometimes used in environmental work because of its increased sensitivity compared with EI even in the SIM mode. However, structural information is lost, and analyte identification based solely on molecular weight is tenuous at best (the molecular weight of the compound can be used as additional evidence for analyte identification).

Early work relied on the use of packed columns, but all modern GC analyses are accomplished using capillary columns with their higher theoretical plate counts and resolution and improved sensitivity. Although a variety of analytical columns have been employed for the GC of triazine compounds, the columns most often used are fused-silica capillary columns coated with 5% phenyl–95% methylpolysiloxane. These nonpolar columns in conjunction with the appropriate temperature and pressure programming and pressure pulse spiking techniques provide excellent separation and sensitivity for the triazine compounds. Typically, columns of 30 m × 0.25-mm i.d. and 0.25-μm film thickness are used of which numerous versions are commercially available (e.g., DB-5, HP-5, SP-5, CP-Sil 8 CB, etc.). Of course, the column selected must be considered in conjunction with the overall design and goals of the particular study.

MS/MS was shown to be more selective than high-resolution MS for the screening of atrazine, simazine, cyanazine, DEA, and DIA in soil.[213] The use of multiple reaction monitoring (MRM) avoided interferences that adversely affected quantitation using the high-resolution mass spectrometry (HRMS) sector instruments. Significant improvement in selectivity was obtained for MS/MS when compared with MS operation using ITD.[214] However, the presence of DIA can interfere with the analysis for DEA when using ITD.[215] This is possibly due to the gas-phase chemistry within the trap, wherein both compounds can fragment to produce the same ion through different mechanisms. The time-scale of the ITD measurement is sufficient to allow re-equilibration of the gas-phase ions or a shift towards ions of another m/z. This is not an issue with quadrupole analyzers. Time-of-flight mass spectrometry (ToFMS) was successfully used for the rapid determination of six triazines (including DEA and DIA) in surface water.[216] Automated spectral peak deconvolution software was required to calculate the spectra from overlapping peaks, and the LOD for the triazines was 4–60 pg. Polar hydroxytriazines not directly amendable to GC analysis were derivatized using *N*-methylbis(trifluoroacetamide) and determined using GC/MS.[217] One trifluoroacetylated derivative was formed for each hydroxy degradate, thus allowing quantitation.

The advantages offered by large-volume injection (LVI) GC are described in recent reports.[218–221] The technique involves the injection of 40–500 μL of the final sample fraction rather than the usual 1–2 μL injected in a typical GC analysis. This allows the use of micro-extraction techniques (micro-LLE, SPE, etc.) with their decreased sample handling and preparation time and lower solvent volume requirements without

sacrificing the sensitivity of the final analysis. The technique can be achieved using on-column injection, programmed temperature vaporization (PTV), or splitless injection with solvent elimination, and each has its unique advantages. Quantitative recoveries were obtained for atrazine, simazine, DEA, terbuthylazine, terbutryn, and metribuzin in groundwater and surface water at the 0.05 $\mu g\,L^{-1}$ concentration level using micro-LLE (1 mL of methyl *tert*-butyl ether) and LVI-PTV-GC/NPD.[222] The injection volume was 200 μL, and the initial water sample volume was 5 mL. The results were similar to those obtained using conventional LLE and analysis using injection volumes of 2 μL into a GC/MS system. Carbopack B (GCB) cartridges were used for the preparation of 1-L water samples (final fraction volume of 500 μL) followed by LVI GC/MS analysis (40-μL injection).[223] The recoveries were quantitative for atrazine, simazine, propazine, DEA, DIA, cyanazine, atraton, and prometon at the 0.10 $\mu g\,kg^{-1}$ concentration level.

7.2 *Liquid chromatography*

Ultraviolet/visible (UV/VIS) and photodiode array (PDA) have been the most often used detectors in LC (see Tables 3 and 4) for the determination of triazine compounds. Ultraviolet (UV) detectors are inexpensive but nonselective. The entire UV/VIS spectrum can be scanned using a PDA detector to identify overlapping or interfering peaks: this increases the selectivity of the analysis, but the technique cannot be considered confirmatory for all analytes, especially when compared with MS. The primary advantage of using LC/UV or LC/PDA methods is that compounds that would normally require derivatization to be determined using GC (e.g., hydroxytriazines) can be determined directly. In addition, sample preparation using SPE is more amenable to LC analysis since a switch to a GC-compatible solvent is not required. The advantages of directly coupling SPE and LC for on-line SPE LC applications were discussed in a previous section. The advantages associated with the coupling of two LC columns were evaluated and reported.[224,225]

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Most reported triazine LC applications are reversed-phase utilizing C-8 and C-18 analytical columns, but there are also a few normal-phase (NH_2, CN) and ion-exchange (SCX) applications. The columns used range from 5 to 25-cm length and from 2 to 4.6-mm i.d., depending on the specific application. In general, the mobile phases employed for reversed-phase applications consist of various methanol and/or acetonitrile combinations in water. The ionization efficiency of methanol and acetonitrile for atmospheric pressure chemical ionization (APcI) applications were compared,[226,227] and based on methanol's lower proton affinity, the authors speculated that more compounds could be ionized in the positive ion mode when using methanol than acetonitrile in the mobile phase.

As with GC, the combination of MS and MS/MS detection with LC adds an important confirmatory dimension to the analysis. Thermospray (TSP) and particle beam (PB) were two of the earlier interfaces for coupling LC and MS, but insufficient fragmentation resulted in a lack of structural information when using TSP, and insufficient sensitivity and an inability to ionize nonvolatile sample components hampered applications using PB. Today, atmospheric pressure ionization (API) dominates the LC/MS field for many environmental applications. The three major variants of API

are APcI, electrospray ionization (ESP), and ionspray (ISP), the last method also being known as high-flow pneumatically assisted electrospray. The APcI interface is sensitive, applicable to a wide range of analyte types (especially low-polarity and nonpolar analytes), and can be used with LC flow rates up to $2\,mL\,min^{-1}$. The ESP and ISP interfaces are particularly sensitive to polar and ionic analytes and produce predominantly quasi-molecular ions ($M+1$ or $M-1$ depending on the charge applied to the capillary). Adducts may also form under certain conditions (e.g., $M+23$ in the presence of Na^+ ion). The primary difference between ESP and ISP is the maximum allowable LC flow rate; in ESP, the total flow should be $\leq 200\,\mu L\,min^{-1}$, whereas in ISP, flow rates as high as $1\,mL\,min^{-1}$ can be handled.[228,229]

The use of collision-induced dissociation (CID) and MS/MS techniques in conjunction with the API interfaces has dramatically impacted the field of environmental analysis. These techniques are now preferred for the determination of triazine compounds in water, soil, crops, etc., owing to the significant improvements in selectivity obtained via the monitoring of precursor–product ion pairs and increased sensitivity due to the reduction of chemical noise.

As an alternative to MS/MS, the feasibility of using liquid chromatography/photolytic dissociation mass spectrometry LC/$h\nu$-MS for the determination of triazines in lettuce and blueberry extracts (prepared using the Luke method) was demonstrated.[230] As the analytes eluted from the LC column, they were mixed post-column with photosensitizer (e.g., acetone) in some experiments prior to entering a photochemical cell (254 or 366 nm) to induce photolytic dissociation. A 150-μL portion of the photolytic cell effluent was admitted to an electrospray/mass spectrometry (ESP/MS) system for analysis. Dehalogenation was the main photolytic-induced process to yield hydroxy- and methoxyatrazine products, whereas dealkylation occurred to a lesser extent. Generally, only two products were formed in methanol and water, but additional ions were formed when sensitizers were used. Blueberry extract was fortified with four triazines (5 ng each), and all four compounds could be identified using LC/$h\nu$-MS analysis. The structurally diagnostic ions differed significantly from those typically obtained in MS/MS analysis using CID. The authors felt that MS/MS still had more selectivity, but this technique was less expensive and could also be used in single-quadrupole LC/MS systems where an in-source CID was not available.

7.3 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) was first reported in 1962, and applications of the technique rapidly increased following the introduction of commercially available instrumentation in the early 1980s due to the ability to determine thermally labile compounds using detection systems more commonly employed with GC. However, few applications of SFC have been published with regard to the determination of triazines. Recently, a chemiluminescence nitrogen detector was used with packed-column SFC and a methanol-modified CO_2 mobile phase for the determination of atrazine, simazine, and propazine.[231] Pressure and mobile phase gradients were used to demonstrate the efficacy of the technique.

7.4 Electrochemical analysis

Owing to their high separation efficiency, the potential for using micellar electrokinetic chromatography (MEKC)[232–234] and capillary zone electrophoresis (CZE)[235] for the determination of triazines was studied. The migration behavior and separation of 13 parent triazine compounds were investigated using MEKC, and complete separation was achieved in 6 min.[236] The coupling of MEKC with ESP/MS for the determination of atrazine, propazine, ametryn, and prometryn was demonstrated.[237] The analytes were separated in a micellar plug prior to entering the electrophoresis buffer that was free of surfactant that allowed ESP/MS analysis without interference from surfactants. Hydroxy degradates of atrazine were determined using both CZE and LC for comparison purposes.[238] The LODs and recoveries at the 0.2 $\mu g\,L^{-1}$ level were comparable, but CZE did reveal some sensitivity to pH, temperature, buffer composition, and capillary dimensions during ruggedness testing. Normal- and reversed-phase electroosmotic flow capillary electrophoresis (CE) was coupled with ESP/MS for the determination of eight triazines.[239] Baseline resolution was not obtained for all eight compounds, but the use of ESP/MS provided on-line compound identification. The composition of the sheath gas in capillary electrophoresis/electrospray/mass spectrometry (CE/ESP/MS) was critical for obtaining resolution comparable to that using capillary electrophoresis/ultraviolet (CE/UV) detection, but there was a sacrifice in sensitivity. At present, resolution and sensitivity cannot be simultaneously optimized.

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7.5 Other techniques

A technique designed for high-speed analysis was recently described in which nanoelectrospray ionization was coupled with gas-phase electrophoresis (GPE).[240] Ions created at atmospheric pressure were separated on the basis of their mobility (dependent on the size and shape of the ion and its charge) through a drift tube. The technique was initially introduced as plasma chromatography and later as a detector in the form of ion mobility spectrometry. The authors chose to use GPE to describe this technique to make a distinction between its use as a separation device rather than a detector. The technique was originally characterized by poor separation efficiency, but modern instrument designs can obtain over 100 000 theoretical plates.[241,242] The low-nanoliter flow rate from the ESP needle in combination with the unique desolvation interface allowed operation under ambient conditions, and the utility of the technique was demonstrated with the analysis of six parent triazines at micromolar concentration levels. The analysis was essentially instantaneous since the time scale for obtaining the spectra was <30 ms.

8 Future directions

Future efforts in the field of environmental analysis will be focused on several fronts, including analyte enrichment and measurement, on-line and on-site techniques, multi-residue methodology, direct injection of aqueous samples into LC/MS/MS

systems, etc. The use of SPE in various formats continues to be the most economical and efficient approach to enrichment of the triazines, because SPE is fast, accurate, precise, and adaptable to automated techniques. Many sorbent types are currently available, including polar, nonpolar, ionic, immunosorbents, molecularly imprinted polymers, etc., that are applicable to analytes of widely varying polarity such as those found amongst parent triazines and their degradates. However, future developments in this area of research will likely result in the creation of new sorbents with novel, unique, and useful selectivities. This, along with creative mixed-mode selectivity, will extend the scope of triazines and other compounds that can be conveniently enriched and also produce final fractions for analysis that contain fewer undesirable sample components, i.e., improved sample purification. For example, immunosorbents and molecularly imprinted polymers can be developed that demonstrate a high degree of selectivity. Although the development of a universal sorbent is unlikely, these improvements will advance the development of multi-residue methodology needed to monitor the environment, especially in terms of water quality. There are some compounds and/or degradates in the environment that are unknown simply because the extraction procedures and/or instrumental operating parameters employed do not account for them.

A wide range of instrumentation can be used in Good Laboratory Practice (GLP) methods as long as specific, pre-established criteria are attained during method validation and ILV ruggedness testing. These criteria partially depend on the objectives and scope of the analytical methodology, and in many cases the least costly instrumental alternative is entirely satisfactory. However, EEC and US EPA regulations also require the development of confirmatory methodology. Thus, many laboratories are finding that method development incorporating MS from the onset is best. Besides, today's bench-top quadrupole and ion-trap MS computer-controlled systems tend to be as easy to use as conventional GC and LC detection systems. The preferred technique, in many cases, is MS/MS owing to its enhanced selectivity and sensitivity. Obviously, mass spectrometric instrumentation is more expensive to purchase and maintain than conventional detection systems, but the goals, study design, number of samples, and other issues must be factored into the overall cost. Hardware and software algorithms continue to improve and expand in scope to allow faster data acquisition and post-run data processing. The trend in multi-channel instrumentation in which one MS/MS system serves several LC instruments will continue to grow in popularity owing to efficiency and cost effectiveness. The tremendous advantages associated with the high resolution obtainable from CZE and MEKC have not been fully realized in the determination of triazines. However, a better understanding and control of certain operating parameters and increased ruggedness when coupled with MS may increase the importance of these techniques in the future.

Well-established, fully automated on-line SPE GC/MS and LC/MS techniques are increasing in robustness and utility. Continued effort along these lines will be rewarded by on-line systems capable of high throughput and reproducibility, and their use is expected to increase. The trend in SPE for on-line applications will be toward smaller sorbent formats and sample volumes and less solvent usage for conditioning, washing, and elution. Interest is increasing in automated on-site techniques for generalized monitoring or screening purposes, and the capability to quantify important analytes at concentration levels of regulatory significance will eventually be routine.

Specifically for triazines in water, multi-residue methods incorporating SPE and LC/MS/MS will soon be available that are capable of measuring numerous parent compounds and all their relevant degradates (including the hydroxytriazines) in one analysis. Continued increases in liquid chromatography/atmospheric pressure ionization tandem mass spectrometry (LC/API-MS/MS) sensitivity will lead to methods requiring no aqueous sample preparation at all, and portions of water samples will be injected directly into the LC column. The use of SPE and GC or LC coupled with MS and MS/MS systems will also be applied routinely to the analysis of more complex sample matrices such as soil and crop and animal tissues. However, the analyte(s) must first be removed from the sample matrix, and additional research is needed to develop more efficient extraction procedures. Increased selectivity during extraction also simplifies the sample purification requirements prior to injection. Certainly, miniaturization of all aspects of the analysis (sample extraction, purification, and instrumentation) will continue, and some of this may involve SFE, subcritical and microwave extraction, sonication, others or even combinations of these techniques for the initial isolation of the analyte(s) from the bulk of the sample matrix.

References

1. A. Gast, E. Knuesli, and H. Gysin, *Experientia,* **11**, 107 (1955).
2. A. Gast, E. Knuesli, and H. Gysin, *Experientia,* **12**, 146 (1956).
3. K.A. Hassall, 'The Biochemistry and Uses of Pesticides,' second edition, VCH, Weinheim (1990).
4. F.M. Ashton and A.S. Crafts, 'The Chemistry and Mode of Action of Herbicides,' Wiley, New York (1981).
5. M. Windholz, S. Budavari, R.F. Blumetti, and E.S. Otterbein (eds.), 'The Merck Index,' tenth edition, Merck, Rahway, NJ (1983).
6. F. Ashton and G. Klingman, 'Weed Science: Principles and Practices,' Wiley, Chichester (1982).
7. A. Kaune, R. Bruggemann, and A. Kettrup, *J. Chromatogr. A,* **805**, 119 (1998).
8. P. Schmitt, T. Poiger, R. Simon, D. Freitag, A. Kettrup, and A.W. Garrison, *Anal. Chem.,* **69**, 2559 (1997).
9. 'Herbicide Handbook,' fourth edition, Weed Science Society of America, Champaign, IL (1979).
10. A.S. Crafts, 'The Chemistry and Mode of Action of Herbicides,' Wiley, New York (1965).
11. S.R. Muller, *Chimica,* **51**, 753 (1997).
12. R.H. Shimabukuro, G.L. Lamoureux, D.S. Frear, and J.E. Bakke, in 'Metabolism of s-Triazines and Its Significance in Biological Systems,' ed. A.S. Tahori, Pesticide Terminal Residues (Supplement to Pure and Applied Chemistry), Butterworth, London (1971).
13. H.O. Esser, G. Dupris, E. Ebert, C. Vogel, and G.J. Macro, in 's-Triazines: Herbicides Chemistry, Degradation, and Mode of Action,' second edition, ed. P.C. Keraney, and D.D. Kaufman, Marcel Dekker, New York, Vol. 1 (1975).
14. D.D. Kaufman and P.C. Kearney, *Residue Rev.,* **32**, 235 (1970).
15. M.C. Gonzales, A.M. Braun, A.B. Prevot, and E. Pelizzetti, *Chemosphere,* **28**, 2121 (1994).
16. L.E. Erickson and K.H. Lee, *Crit. Rev. Environ. Control,* **19**, 1 (1989).
17. M. Knutson, G. Nilve, L. Mathiasson, and J. Jonson, *J. Chromatogr. A,* **793**, 257 (1996).
18. M. Fielding, D. Barcelo, A. Helweg, S. Galassi, L. Torstenson, P. van Zoonen, R. Wolter, and G. Angeletti, in 'Pesticides in Ground and Drinking Water (Water Pollution Research Report 27),' Commission of the European Communities, Brussels, pp. 1–136 (1992).
19. EPA 822-B-00-001, US Environmental Protection Agency, Drinking Water Standards and Health Advisories, Office of Water, Washington, DC (2000).

DropBooks

20. J.L. Tadeo, C. Sanchez-Brunete, A.I. Garcia-Valcarcel, L. Martinez, and R.A. Perez, *J. Chromatogr. A,* **754**, 347 (1996).
21. J.R. Dean, G. Wade, and I.J. Barnabas, *J. Chromatogr. A,* **733**, 295 (1996).
22. H. Sabik, R. Jeannot, and B. Rondeau, *J. Chromatogr. A,* **885**, 217 (2000).
23. E.A. Scribner, E.M. Thurman, and L.R. Zimmerman, *Sci. Total Environ.,* **248**, 157 (2000).
24. T.P.J. Linsinger, M. Fuhrer, W. Kandler, and R. Schuhmacher, *Analyst,* **126**, 211 (2001).
25. EPA 500 Series, Method 507, Revision 2.0, US Environmental Protection Agency, Washington, DC (1989).
26. G.D. Foster, P.M. Gates, W.T. Foreman, S.W. McKenzie, and F.A. Rinella, *Environ. Sci. Technol.,* **27** (1993).
27. C. Charreteur, N. Kerbaol, and J.J. Peron, *Analusis,* **24**, 336 (1996).
28. N.H. Spliid and B. Koppen, *J. Chromatogr.,* **736**, 105 (1996).
29. A. Gong and C. Ye, *J. Chromatogr. A,* **827**, 57 (1998).
30. C. Molina, G. Durand, and D. Barcelo, *J. Chromatogr. A,* **712**, 113 (1995).
31. B.M. Mahara, J. Borossay, and K. Torkos, *Microchem. J.,* **58**, 31 (1998).
32. V.L. Lanchote, P.S. Bonato, A.L. Cerderia, N.A.G. Santos, D. De Carvalho, and M.A. Gomes, *Water Air Soil Pollut.,* **118**, 329 (2000).
33. C. Yrieix, C. Gonzalez, J.M. Deroux, C. Lacoste, and J. Leybros, *Water Res.,* **8**, 1791 (1996).
34. M.J. Fernandez, C. Garcia, R.J. Carcia-Villanove, and J.A. Gomez, *J. Agric. Food Chem.,* **44**, 1790 (1996).
35. R. Alzaga, G. Durand, D. Barcelo, and J.M. Bayona, *Chromatographia,* **38**, 502 (1994).
36. R.A. Yokley and M.W. Cheung, *J. Agric. Food Chem.,* **48**, 4500 (2000).
37. Z. Cai, M.L. Gross, and R.F. Spalding, *Anal. Chim. Acta,* **304**, 67 (1995).
38. V. Pacakova, K. Stulik, and J. Jiskra, *J. Chromatogr.,* **754**, 17 (1996).
39. T.L. Potter, T. Carpenter, R. Putnam, K. Reddy, and J.M. Clark, *J. Agric. Food Chem.,* **39**, 2184 (1991).
40. K. Vandecasteele, I. Gaus, W. Debreuck, and K. Walraevens, *Anal. Chem.,* **72**, 3093 (2000).
41. R.E. Majors, *LC-GC,* **16**, 8 (1998).
42. E.M. Thurman and M. Mills, 'Solid-phase Extraction: Principles and Practice,' Wiley, New York (1998).
43. M.-C. Hennion, C. Cau-Dit-Coumes, and V. Pichon, *J. Chromatogr.,* **823**, 147 (1998).
44. M.-C. Hennion, *J. Chromatogr. A,* **856**, 3 (1999).
45. R.E. Majors, *LC-GC,* **4**, 972 (1986).
46. L.M. Davi, M. Baldi, L. Penazzi, and M. Liboni, *Pestic. Sci.,* **25**, 63 (1992).
47. J.C. Molto, B. Lejeune, P. Prognon, and D. Pradeau, *Int. J. Environ. Anal. Chem.,* **54**, 81 (1994).
48. E. Viana, M.J. Redondo, G. Font, and J.C. Molto, *J. Chromatogr.,* **733**, 267 (1996).
49. E. Bolygo and N.C. Atreya, *Fresenius' J. Anal. Chem.,* **339**, 423 (1991).
50. M.J.M. Wells and J.L. Michael, *J. Chromatogr. Sci.,* **25**, 345 (1987).
51. C. Crescenzi, A. Di Corcia, E. Guerriero, and R. Samperi, *Environ. Sci. Technol.,* **31**, 479 (1997).
52. R. Carbias-Martinez, E. Rodriquez-Gonzalo, M.E. Fernandez-Laespada, and F.J.S. San Roman, *J. Chromatogr. A,* **869**, 471 (2000).
53. A. Aguilar, F. Borrull, and R.M. Marce, *J. Chromatogr. A,* **771**, 221 (1997).
54. T.A. Albanis and D.G. Hela, *J. Chromatogr. A,* **707**, 283 (1995).
55. R. Carabias-Martinez, E. Rodrigues-Gonzalo, J. Dominquez-Alvarez, and J. Hernandez-Mendez, *J. Chromatogr. A,* **869**, 451 (2000).
56. R. Carabias-Martinez, E. Rodriquez-Gonzalo, A.I. Munoz-Dominquez, J. Dominquez-Alvarez, and J. Hernandez-Mendez, *J. Chromatogr. A,* **733**, 349 (1996).
57. C. Crescenzi, A. Di Corcia, M.D. Mabdouly, and R. Samperi, *Environ. Sci. Technol.,* **29**, 2185 (1995).
58. J. Dugay, C. Miege, and M.-C. Hennion, *J. Chromatogr. A,* **795**, 27 (1998).
59. M. Psathaki, E. Manoussridou, and E.G. Stephanou, *J. Chromatogr. A,* **667**, 241 (1994).
60. T.K. Choudbury, K.O. Gerhardt, and T.P. Mawhinney, *Environ. Sci. Technol.,* **30**, 3259 (1996).
61. C. Aguilar, I. Ferrer, F. Borrull, R.M. Marce, and D. Barcelo, *J. Chromatogr. A,* **794**, 147 (1998).
62. T.S. Thompson and B.D. Miller, *Chemosphere,* **36**, 2867 (1998).

63. G.M.F. Pinto and I.C.S.F. Jardim, *J. Chromatogr. A,* **869**, 463 (2000).
64. J.-B. Fournier, M. El Hourch, and J. Fournier, *Analusis,* **27**, 726 (1999).
65. D.A. Cassada, R.F. Spalding, Z. Cai, and M.L. Gross, *Anal. Chim. Acta,* **287**, 7 (1994).
66. E.M. Thurman, M. Meyer, M. Pomes, C.A. Perry, and A.P. Schwab, *Anal. Chem.,* **62**, 2043 (1990).
67. I. Ferrer, D. Barcelo, and E.M. Thurman, *Anal. Chem.,* **71**, 1009 (1999).
68. I. Vassilakis, D. Tsipi, and M. Scoullos, *J. Chromatogr. A,* **823**, 49 (1998).
69. A. Di Corcia, C. Crescenzi, E. Guerriero, and R. Samperi, *Environ. Sci. Technol.,* **31**, 1658 (1997).
70. D. Barcelo, G. Durand, V. Bouvot, and M. Nielen, *Environ. Sci. Technol.,* **27**, 217 (1993).
71. R.N. Lerch, W.W. Donald, Y.-X Li, and E.E. Alberts, *Environ. Sci. Technol.,* **29**, 2759 (1995).
72. H. Sabik, S. Cooper, P. Lafrance, and J. Fournier, *Talanta,* **42**, 717 (1995).
73. H. Sabik, *Int. J. Environ. Anal. Chem.,* **72**, 113 (1998).
74. H. Sabik and R. Jeannot, *J. Chromatogr. A,* **818**, 197 (1998).
75. B. Nouri, G. Toussaint, P. Chambon, and R. Chambon, *Analyst,* **120**, 2683 (1995).
76. A. Di Corcia, R. Samperi, A. Marcomini, and S. Stelluto, *Anal. Chem.,* **65**, 907 (1993).
77. H. Farber, K. Nick, and H.F. Scholer, *Fresenius' J. Anal. Chem.,* **350**, 145 (1994).
78. Z. Cai, V.M. Sadagopa Ramanujam, M.L. Gross, S.J. Monson, D.A. Cassada, and R.F. Spalding, *Anal. Chem.,* **66**, 4202 (1994).
79. R.N. Lerch and W.W. Donald, *J. Agric. Food Chem.,* **42**, 922 (1994).
80. M. Berg, S.R. Muller, and R.P. Schwarzenbach, *Anal. Chem.,* **67**, 1860 (1995).
81. D.T. Kubilius and R.J. Bushway, *J. Chromatogr. A,* **793**, 349 (1998).
82. J. Patsias and E. Papadopoulou-Mourkidou, *J. Chromatogr. A,* **740**, 83 (1996).
83. A. Saez, D.G. de Barreda, M. Gamon, J.G. de la Cuadra, E. Lorenzo, and C. Peris, *J. Chromatogr. A,* **721**, 107 (1996).
84. Z. Cai, R.L. Cerny, and R.F. Spalding, *J. Chromatogr. A,* **753**, 243 (1996).
85. T.D. Bucheli, F.C. Grusbler, S.R. Muller, and R. Schwartzenbach, *Anal. Chem.,* **69**, 1569 (1997).
86. R. Loos and R. Niessner, *J. Chromatogr. A,* **835**, 217 (1999).
87. R.J.C.A. Steen, A.C. Hogenboom, P.E.G. Leonards, R.A.L. Peerboom, W.P. Cofino, and U.A.Th. Brinkman, *J. Chromatogr. A,* **857**, 157 (1999).
88. E. Turiel, P. Fernandez, C. Perez-Conde, and C. Camara, *J. Chromatogr. A,* **872**, 299 (2000).
89. S.Y. Panshin, D.S. Carter, and E.R. Bayless, *Environ. Sci. Technol.,* **34**, 2131 (2000).
90. D.A. Azevedo, S. Lacorte, P. Viana, and D. Barcelo, *Chromatographia,* **53**, 113 (2001).
91. D. Giraud, A. Ventura, V. Camel, A. Bermond, and P. Arpino, *J. Chromatogr. A,* **777**, 115 (1997).
92. R.A. McLaughlin and B.S. Johnson, *J. Chromatogr. A,* **790**, 161 (1997).
93. I. Tolosa, B. Douy, and F.P. Carvalho, *J. Chromatogr. A,* **864**, 121 (1999).
94. E. Noroozian, F.A. Maris, M.W.F. Nielen, R.W. Frei, G.J. de Jong, and U.A.Th. Brinkman, *J. High Resolut. Chromatogr.,* **10**, 17 (1987).
95. D.F. Hagan, C.G. Markell, G.A. Schmidt, and D.D. Blevins, *Anal. Chim. Acta,* **236**, 157 (1990).
96. M.-C. Hennion, *Analusis,* **26**, 131 (1998).
97. M. Masque, R.M. Marce, and F. Borrull, *J. Chromatogr. A,* **793**, 257 (1998).
98. G.R. Mills, *J. Chromatogr. A.,* **813**, 63 (1998).
99. V. Pichon and M.-C. Hennion, *J. Chromatogr. A,* **665**, 269 (1994).
100. H. Prosen, L. Zupancic-Kralj, and J. Marsel, *J. Chromatogr. A,* **704**, 121 (1995).
101. C. Aguilar, F. Borrull, and R.M. Marce, *J. Chromatogr. A,* **754**, 77 (1996).
102. J. Slobodnik, A.C. Hogenboom, J.J. Vreuls, J.A. Rontree, B.L.M. van Baar, W.M.A. Niessen, and U.A.Th. Brinkman, *J. Chromatogr. A,* **741**, 59 (1996).
103. G.W. Somsen, I. Jagt, C. Gooijer, N.H. Velthorst, U.A.Th. Brinkman, and T. Visser, *J. Chromatogr. A,* **756**, 145 (1996).
104. S. Dupas, P. Scribe, and J.F. Dubernet, *J. Chromatogr. A,* **737**, 117 (1996).
105. W.A. Minnaard, A.C. Hogenboom, U.K. Malmqvist, P. Manini, W.M.A. Niessen, and U.A.Th. Brinkman, *Rapid Commun. Mass Spectrom.,* **10**, 1569 (1996).
106. P. Onnerfjord, D. Barcelo, J. Emneus, L. Gorton, and G. Marko-Varga, *J. Chromatogr. A,* **737**, 35 (1996).
107. E. Pocurull, C. Aguilar, F. Borrull, and R.M. Marce, *J. Chromatogr. A,* **818**, 85 (1998).

DropBooks

108. N. Masque, M. Galia, R.M. Marce, and F. Borrull, *J. High Resolut. Chromatogr.,* **22**, 547 (1999).
109. N. Masque, M. Galia, R.M. Marcee, and F. Borrull, *J. Chromatogr. A,* **803**, 127 (1998).
110. I. Ferrer, E.M. Thurman, and D. Barcelo, *Environ. Sci. Technol.,* **34**, 714 (2000).
111. N. Masque, R.M. Marcee, and F. Borrull, *Trends Anal. Chem.,* **17**, 384 (1998).
112. V. Pichon, H. Rogniaux, N. Fischer-Durand, S. Ben Rejeb, F. Le Goffic, and M.-C. Hennion, *Chromatographia,* **45**, 289 (1997).
113. I. Ferrer, V. Pichon, M.-C. Hennion, and D. Barcelo, *J. Chromatogr. A,* **777**, 91 (1997).
114. I. Ferrer, M.-C. Hennion, and D. Barcelo, *Anal. Chem.,* **69**, 4508 (1997).
115. J.G. Rollag, M. Beck-Westermeyer, and D.S. Hage, *Anal. Chem.,* **68**, 3631 (1996).
116. J. Dalluge, T. Hankemeier, R.J.J. Vreuls, and U.A.Th. Brinkman, *J. Chromatogr. A,* **830**, 377 (1999).
117. A. Marx, Th. Giersch, and B. Hock, *Anal. Lett.,* **28**, 267 (1995).
118. Th. Hankemeier, P.C. Steketee, J.J. Vreuls, and U.A.Th. Brinkman, *J. Chromatogr. A,* **750**, 161 (1996).
119. A.C. Hogenboom, U.K. Malmqvist, K. Nolkrantz, J.J. Vreuls, and U.A.Th. Brinkman, *J. Chromatogr. A,* **759**, 55 (1997).
120. A.C. Hogenboom, J. Slobodnik, J.J. Vreuls, J.A. Rontree, B.L.M. vab Baar, W.M.A. Niessen, and U.A.Th. Brinkman, *Chromatographia,* **42**, 506 (1996).
121. W.A. Minnard, J. Slobodnik, J.J. Vreuls, K.-P. Hupe, and U.A.Th. Brinkman, *J. Chromatogr. A,* **696**, 333 (1995).
122. A.C. Hogenboom, W.M.A. Niessen, and U.A.Th. Brinkman, *J. Chromatogr. A,* **794**, 201 (1998).
123. S.A. Piletsky, E.V. Piletskaya, and A.V. El'skaya, *Anal. Lett.,* **30**, 445 (1997).
124. K. Yano and I. Karube, *Trends Anal. Chem.,* **18**, 199 (1999).
125. S.A. Piletsky, E.V. Piletskaya, A.V. Elgersma, K. Yano, I. Karube, Yu.P. Parhometz, and A.V. El'skaya, *Biosens. Bioelectron.,* **10**, 959 (1995).
126. N. Masque, R.M. Marce, and F. Borrull, *Trends Anal. Chem.,* **20**, 477 (2001).
127. B. Sellergren, *Anal. Chem.,* **66**, 1578 (1994).
128. J.H.G. Steinke, I.R. Dunkin, and D.C. Sherrington, *Macromolecules,* **29**, 407 (1996).
129. M.E. Cooper, B.P. Hoag, and D.L. Gin, *Polym. Prepr.,* **38**, 209 (1997).
130. J. Matsui, Y. Tachibana, and T. Takeuchi, *Anal. Commun.,* **35**, 225 (1998).
131. K. Ensing, C. Berggren, and R.E. Majors, *LC-GC,* **19**, 942 (2001).
132. R.C. Martinez, E.R. Gonzalo, M.P. Santiago Toribio, and J. Hernandez Mendez, *Anal. Chimi. Acta,* **321**, 147 (1996).
133. R. Eisert and K. Levsen, *J. Chromatogr. A,* **737**, 59 (1996).
134. C. Aguilar, S. Penalver, E. Pocurull, F. Borrull, and R.M. Marce, *J. Chromatogr. A,* **795**, 105 (1998).
135. R. Ferrari, T. Nilsson, R. Arena, P. Arlati, G. Bartolucci, R. Basla, F. Cioni, G. Del Carlo, P. Dellavedove, E. Fattore, M. Fungi, C. Grote, M. Guidotti, S. Morgillo, L. Muller, and M. Volante, *J. Chromatogr. A,* **795**, 371 (1998).
136. I.J. Barnabas, J.R. Dean, I.A. Fowlis, and S.P. Owen, *J. Chromatogr. A,* **705**, 305 (1995).
137. I.J. Barnabas, J.R. Dean, S.M. Hitchen, and S.P. Owen, *J. Chromatogr. Sci.,* **32**, 547 (1994).
138. R. Alzaga, G. Durand, D. Barcelo, and J.M. Bayona, *Chromatographia,* **38**, 502 (1994).
139. S. Chiron, A.F. Alba, and D. Barcelo, *Environ. Sci. Technol.,* **27**, 2352 (1993).
140. D.J. Munch and C.P. Frebis, *Environ. Sci. Technol.,* **26**, 921 (1992).
141. M.W. Cheung and R.A. Yokley, Report ABR-94094, Syngenta, Greensboro, NC (1996).
142. R. A. Yokley, Report 383-97, Syngenta, Greensboro, NC (1997).
143. C. Crescenzi, A. Di Corcia, M.D. Madbouly, and R. Samperi, *Environ. Sci. Technol.,* **29**, 2185 (1995).
144. I. Liska and K. Bilikova, *J. Chromatogr. A,* **795**, 61 (1998).
145. C. Aguilar, I. Ferrer, F. Borrull, R.M. Marce, and D. Barcelo, *Anal. Chim. Acta,* **386**, 237 (1999).
146. U.A.Th. Brinkman, J. Slobodnik, and J.J. Vreuls, *Trends Anal. Chem.,* **13**, 373 (1994).
147. G. Sposito, L. Martin-Neto, and A. Yang, *J. Environ. Qual.,* **25**, 1203 (1996).
148. C.G. Zambonin, F. Catucci, and F. Palmisano, *Analyst,* **123**, 2825 (1998).
149. G. Durand, R. Forteza, and D. Barcelo, *Chromatographia,* **28**, 597 (1989).

150. H. Weil and K. Haberer, *Fresenius' J. Anal. Chem.,* **339**, 405 (1991).
151. A. Hrdlicka and J. Dolinova, *J. Liq. Chromatogr. Relat. Technol.,* **24**, 721 (2001).
152. K. Bester and H. Huhnerfuss, *Fresenius' J. Anal. Chem.,* **358**, 630 (1997).
153. R.A. Yokley, L.C. Mayer, R. Rezzaiyan, M.E. Manuli, and M.W. Cheung, *J. Agric. Food Chem.*, **48**, 3352 (2000).
154. R.M. Johnson, F. Halaweish, and J.J. Fuhrmann, *J. Liq. Chromatogr.,* **15**, 2941 (1992).
155. M.M. Galera, J.L.M. Vidal, and A.G. Frenich, *Anal. Lett.,* **27**, 807 (1994).
156. M.M. Galera, J.L.M. Vidal, A.G. Frenich, and P.P. Vazquez, *J. AOAC Int.,* **78**, 423 (1995).
157. H.J. Turin and R.S. Bowman, *J. Environ. Qual.,* **22**, 332 (1993).
158. M.C. Gennaro, D. Giacosa, and C. Baglietto, *J. Liq. Chromatogr. Relat. Technol.,* **19**, 911 (1996).
159. H. Sabik, S. Cooper, P. Lafrance, and J. Fournier, *Talanta,* **5**, 717 (1995).
160. M.S. Mills and E.M. Thurman, *Anal. Chem.,* **64**, 1985 (1992).
161. E.G. Cotterill, *Pestic. Sci.,* **11**, 23 (1980).
162. H. Roseboon and H.A. Herbold, *J. Chromatogr.,* **202**, 431 (1980).
163. B.G. Tweedy and R.A. Kahrs, in 'Analytical Methods for Pesticides and Plant Growth Regulators, New and Updated Methods,' ed. G. Zweig, Academic Press, New York, Vol. X, p. 515 (1978).
164. R. Schewes, S. Wust, J.L-v. Gleissenthall, F.X. Maidl, A. Sub, B. Hock, and G. Fleschbeck, *Anal. Lett.,* **27**, 487 (1994).
165. T.M. Vickrey, D.L. Karlesky, and G.L. Blackmer, *J. AOAC,* **63**, 506 (1980).
166. W.D. Hormann, G. Formica, K. Ramsteiner, and D.O. Eberle, *J. AOAC,* **55**, 1031 (1972).
167. A.O. Schwab, P. Splichal, and L.S. Sonon, In 'Application of Agricultural Analysis in Environmental Studies,' ASTM STP 1162, ed. K.B. Hoddinott and T.A. O'Shay, ASTM, Philadelphia, p. 86 (1993).
168. V. Laabs, W. Amelung, and W. Zech, *J. Environ. Qual.,* **28**, 1778 (1999).
169. P.L. del Valle and J.O. Nelson, *Arch. Environ. Contam. Toxicol.,* **27**, 375 (1994).
170. W. Dedek, L. Weil, and R. Niessner, *Fresenius' Environ. Bull.,* **5**, 241 (1996).
171. C. Sanchez-Brunete, R.A. Perez, E. Miguel, and J.L. Tadeo, *J. Chromatogr. A,* **823**, 17 (1998).
172. M.J. Redondo, M.J. Ruiz, R. Boluda, and G. Font, *Chromatographia,* **36**, 187 (1993).
173. H. Prosen and L. Zupancic-Kralj, *Acta Chim. Slov.,* **45**, 19 (1998).
174. I.J. Barnabas, J.R. Dean, I.A. Fowlis, and S.P. Owen, *Analyst,* **120**, 1897 (1995).
175. V. Lopez-Avila, R. Young, and W.F. Beckert, *Anal. Chem.,* **66**, 1097 (1994).
176. R. Hoogerbrugge, C. Molins, and R.A. Baumann, *Anal. Chim. Acta,* **348**, 247 (1997).
177. C. Molins, E.A. Hogendoorn, H.A.G. Heusinkveld, A.C. van Beuzekom, P. van Zoonen, and R.A. Baumann, *Chromatographia,* **48**, 450 (1998).
178. Z. Knez, A. Rizner-Hras, K. Kokot, and D. Bauman, *Fluid Phase Equil.,* **152**, 95 (1998).
179. S. Papilloud and W. Haerdi, *Chromatographia,* **38**, 514 (1994).
180. S. Papilloud and W. Haerdi, *Chromatographia,* **40**, 705 (1995).
181. M. Kolb and B. Englert, *Acta Hydrochim. Hydrobiol.,* **24**, 277 (1996).
182. T. Yarita, Y. Horimoto, A. Nomura, and S. Gonda, *Chromatographia,* **42**, 551 (1996).
183. R.J. Schneider, *Agribiol. Res.,* **48**, 193 (1995).
184. M.E. Shows and S.V. Olesik, *J. Chromatogr. Sci.,* **38**, 399 (2000).
185. A. Di Corcia, A.B. Caracciolo, C. Crescenzi, G. Giuliano, S. Murtas, and R. Samperi, *Environ. Sci. Technol.,* **33**, 3271 (1999).
186. M.S.S. Curren and J.W. King, *Anal. Chem.,* **73**, 740 (2001).
187. C.G. Zambonin and F. Palmisano, *J. Chromatogr. A,* **874**, 247 (2000).
188. M.J. Ruiz, M.J. Redondo, and G. Font, *J. Chromatogr. A,* **776**, 348 (1997).
189. V. Camel, A. Tambute, and M. Caude, *J. Chromatogr.,* **642**, 263 (1993).
190. S.B. Hawthorne, D.J. Miller, M.D. Burford, J.J. Langenfeld, S. Eckert-Tilotta, and P.K. Louie, *J. Chromatogr.,* **642**, 301 (1993).
191. T.L. Chester, J.D. Pinkston, and D.E. Raynie, *Anal. Chem.,* **66**, 106R (1994).
192. M.H. Liu, S. Kapila, K.S. Nam, and A.A. Elseewi, *J. Chromatogr.,* **639**, 151 (1993).
193. C. Mougin, J. Dubroca, and E. Barriuso, *J. High Resolut. Chromatogr.,* **19**, 700 (1996).
194. F.M. Lancas and M.A. Ruggiero, *J. Microcol. Sep.,* **12**, 61 (2000).
195. J.R. Pardue and R. Bong, *ACS Symp. Ser.,* **683**, 123 (1998).

DropBooks

196. J.R. Pardue, *J. AOAC Int.,* **78**, 856 (1995).
197. P.D. Johnson, D.A. Rimmer, and R.H. Brown, *J. Chromatogr. A,* **765**, 3 (1997).
198. A-J. Gong, C-M. Ye, Z-F. Lei, and J-A. Liu, *J. Liq. Chromatogr. Relat. Technol.,* **22**, 2535 (1999).
199. J.F. Lawrence, C. Menard, M-C. Hennion, V. Pichon, F. LeGoffic, and N. Durand, *J. Chromatogr. A,* **752**, 147 (1996).
200. D.C. Holland, R.K. Munns, J.E. Roybal, J.A. Hurlbut, and A.R. Long, *J. AOAC Int.,* **78**, 1067 (1995).
201. M.S.S. Curren and J.W. King, *J. Agric. Food Chem.,* **49**, 2175 (2001).
202. J.W. Pensabene, W. Fiddler, and D.J. Donoghue, *J. Agric. Food Chem.,* **48**, 1668 (2000).
203. R. Garcia-Repetto, I. Garrido, and M. Repetto, *J. AOAC Int.,* **79**, 1423 (1996).
204. M. Hack, S. Nitz, and H. Parlar, *Adv. Food Sci.,* **18**, 40 (1996).
205. G. Catenacci, F. Barbieri, M. Bersani, A. Ferioli, D. Cottica, and M. Maroni, *Toxicol. Lett.,* **69**, 217 (1993).
206. A.D. Lucas, A.D. Jones, M.H. Goodrow, S.G. Saiz, C. Blewett, J.N. Seiber, and B.D. Hammock, *Chem. Res. Toxicol.,* **6**, 107 (1993).
207. L.H. Bodalbhai, R.A. Yokley, and M.W. Cheung, *J. Agric. Food Chem.,* **46**, 161 (1998).
208. T. Kumazawa, K. Sato, H. Seno, and O. Suzuki, *Forensic Sci. Int.,* **54**, 159 (1992).
209. J. Pommery, M. Mathieu, D. Mathieu, and M. Lhermitte, *J. Chromatogr. A,* **526**, 569 (1990).
210. B.J. Wienhold, A.M. Sadeghi, and T.J. Gish, *J. Environ. Qual.,* **22**, 162 (1993).
211. M. Millet, H. Wortham, A. Sanusi, and P. Mirabel, *Arch. Environ. Contam. Toxicol.,* **31**, 543 (1996).
212. A. Sanusi, M. Millet, H. Wortham, and P. Mirabel, *Analusis,* **25**, 302 (1997).
213. G. Durand, P. Gille, D. Fraisse, and D. Barcelo, *J. Chromatogr. A,* **603**, 175 (1992).
214. P. Sandra, J. Beltran, and F. David, *J. High Resolut. Chromatogr.,* **18**, 545 (1995).
215. M.L. Magnuson, T.F. Speth, and C.A. Kelty, *J. Chromatogr. A,* **868**, 115 (2000).
216. R.J.J. Vruels, J. Dalluge, and U.A.Th. Brinkman, *J. Microcol. Sep.,* **11**, 663 (1999).
217. R. Frassanito, G. De Socio, D. Laura, and D. Rotilo, *J. Agric. Food Chem.,* **44**, 2282 (1996).
218. J. Bertran, F.J. Lopez, M. Forcada, and F. Hernandez, *Anal. Chim. Acta,* **356**, 125 (1997).
219. J. Beltran, F.J. Lopez, M. Forcada, and F. Hernandez, *Chromatographia,* **44**, 274 (1997).
220. C. Charreteur, R. Colin, D. Morin, and J.J. Peron, *Analusis,* **26**, 8 (1998).
221. J. Teske, J. Efer, and W. Engewald, *Chromatographia,* **47**, 35 (1998).
222. M. Forcada, J. Beltran, F.J. Lopez, and F. Hernandez, *Chromatographia,* **51**, 362 (2000).
223. R. Jeannot, H. Sabik, L. Amalric, E. Sauvard, S. Proulx, and B. Rondeau, *Chromatographia,* **54**, 236 (2001).
224. F. Hernandez, C. Hidalgo, J.V. Sancho, and F.J. Lopez, *J. Chromatogr. A,* **778**, 171 (1997).
225. F. Hernandez, C. Hidalgo, J.V. Sancho, and F.J. Lopez, *Anal. Chem.,* **70**, 3322 (1998).
226. R.B. Geerdink, A. Kooistra-Sijpersma, J. Tiesnitsch, P.G.M. Kienhuis, and U.A.Th. Brinkman, *J. Chromatogr. A,* **863**, 147 (1999).
227. P.G.M. Kienhuis and R.B. Geerdink, *Trends Anal. Chem.,* **19**, 249 (2000).
228. I. Ferrer and D. Barcelo, *Analusis,* **26**, 118 (1998).
229. R. Jeannot and E. Sauvard, *Analusis,* **27**, 271 (1999).
230. D.A. Volmer, *J. Chromatogr. A,* **794**, 129 (1998).
231. H. Shi, L.T. Taylor, and E.M. Fujinari, *J. Chromatogr. A,* **757**, 183 (1997).
232. W.M. Nelson and C.S. Lee, *Anal. Chem.,* **68**, 3265 (1996).
233. L. Yang, A.K. Harrata, and C.S.Lee, *Anal. Chem.,* **69**, 1820 (1997).
234. A. Farran, S. Ruiz, C. Serra, and M. Aguilar, *J. Chromatogr. A,* **737**, 109 (1996).
235. P. Schmitt, A.W. Garrison, D. Freitag, and A. Kettrup, *J. Chromatogr. A,* **723**, 169 (1996).
236. C.-E. Lin, C.-C. Hsueh, T.-Z. Wang, T.-C. Chiu, and Y.-C. Chen, *J. Chromatogr. A,* **835**, 197 (1999).
237. W.M. Nelson, Q. Tang, A.K. Harrata, and C.S. Lee, *J. Chromatogr. A,* **749**, 219 (1996).
238. H. Stutz, K. Pitterschatscher, and H. Malissa, Jr, *Mikrochim. Acta,* **128**, 107 (1998).
239. C.-Y. Tsai, Y.-R. Chen, and G.-R. Her, *J. Chromatogr. A,* **813**, 379 (1998).
240. D.C. Collins and M.L. Lee, *Fresenius' J. Anal. Chem.,* **369**, 225 (2001).
241. G.R. Asbury and H.H. Hill, Jr, *J. Microcol. Sep.,* **12**, 172 (2000).
242. C. Wu, W.F. Siems, and H.H. Hill, Jr, *Anal. Chem.,* **72**, 396 (2000).

Diphenyl ethers

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1 Introduction

Diphenyl ethers are both systemic and contact herbicides and are used for the selective control of annual broad-leaved weeds and grasses in a variety of crops (such as soybeans, maize, rice, wheat, barley, peanuts, cotton, onions and ornamental trees) under different application scenarios. This class of herbicides contains a diphenyl ether moiety as the core substructure. Acifluorfen, bifnox, chlomethoxyfen, chlornitrofen, fluoroglycofen-ethyl and fomesafen, etc., are representative compounds of the diphenyl ether herbicides (Figure 1).

The mode of action of diphenyl ether herbicides is the inhibition of protoporphyrinogen oxidase (PPO) and this inhibitory action is light-activated. The herbicides absorbed by plants inhibit PPO in the system of porphyrin–chlorophyll synthesis, and the chlorophyll precursor protoporphyrinogen IX is accumulated in the plants. The excess protoporphyrinogen IX in the thylakoid membrane leads to oxidation of protoporphyrin IX, which is the strong photosensitizer for producing singlet oxygen. The reactive singlet oxygen disrupts the plasma membrane and the breakdown of membrane unsaturated fatty acids, resulting in the loss of chlorophyll and carotenoids and in leaky membranes.[1]

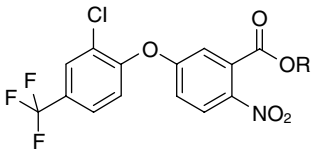
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The diphenyl ether herbicides are nonvolatile compounds, generally very lipophilic and insoluble in water. Solubility in water and octanol–water partition coefficients ($\log K_{ow}$) of the various diphenyl ether herbicides range from 120 mg L^{-1} (acifluorfen) to 0.16 mg L^{-1} (oxyfluorfen) and from 2.9 (fomesafen) to 5.4 (acifluorfen), respectively. Diphenyl ether herbicides are stable in an acidic or alkaline condition, but some compounds are gradually degraded under the sunlight.[2]

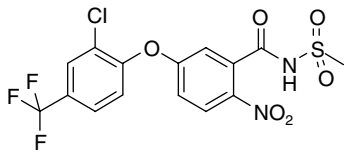
Because of the limited root uptake and slow rate of systemic translocation, the diphenyl ether herbicide residues detected in the aerial plant portion are low.

In Japan, bifenox is the only registered diphenyl ether herbicide. The tolerance and/or maximum residue limits (MRLs) are established at 0.1 mg kg^{-1} for cereals such as rice grain, barley and wheat, and 0.05 mg kg^{-1} for potatoes (Ministry of Health, Labour and Welfare, Japan). The California Department of Food and Agriculture (CDFA) established the minimum detectable quantity of diphenyl ether herbicides at 0.1 mg kg^{-1} for bifenox, nitrofen and oxyfluorfen.[3]

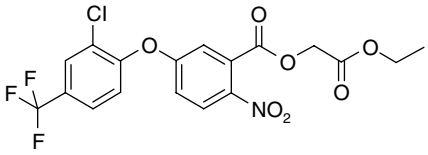
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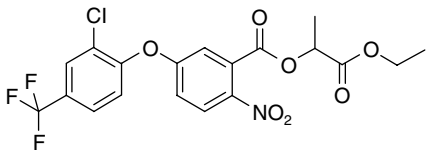
R=Na, Acifluorfen-sodium;
R=CH_3,Acifluorfen-methyl;
R=C_2H_5, Acifluorfen-ethyl



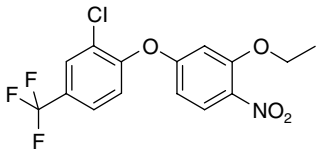
Fomesafen



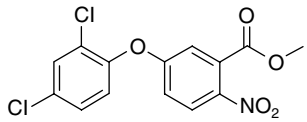
Fluoroglycofen-ethyl



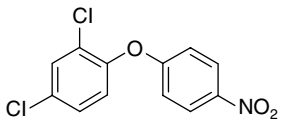
Lactofen



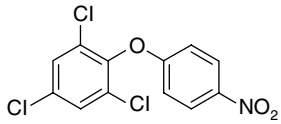
Oxyfluorfen



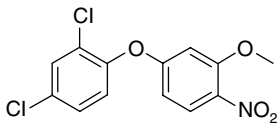
Bifenox



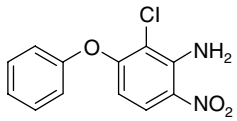
Nitrofen



Chlornitrofen



Chlomethoxyfen



Aclonifen

Figure 1 Structures of diphenyl ether herbicides

A residue analytical method for diphenyl ethers in soil and water samples and in crop samples has been developed. The basic principle of the residue method consists of the following steps: extraction from the samples with acetone or other organic solvents, purification using liquid–liquid partition and column chromatography including solid-phase extraction (SPE), and quantitative analysis by gas chromatography/electron capture detection (GC/ECD), gas chromatography/nitrogen–phosphorus detection (GC/NPD) or high-performance liquid chromatography (HPLC).

2 Analytical methodology for plant materials

2.1 Nature of the residues

Diphenyl ethers in the soil are absorbed by roots with limited translocation generally to the foliage. Low levels of herbicide residues can be expected when the compound is used in accordance with good agricultural practice. The parent diphenyl ether compound is defined as the residue of analytical and regulatory concern.

2.2 Analytical method

A 20-g homogenized cereal or vegetable sample is extracted with an organic solvent such as acetone. After filtration, the solvent extract is concentrated by rotary evaporation to about 20 mL, below 40 °C. The residue is transferred with 5% sodium chloride solution and partitioned twice with n-hexane. The n-hexane extracts are dried by anhydrous sodium sulfate, which is subjected to a cleanup procedure by Florisil or silica gel column chromatography. The eluate is concentrated to dryness and the residue is dissolved in an appropriate amount of acetone for GC/ECD (Ministry of the Environment, Japan).

2.2.1 Extraction

(1) Vegetables

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20-g of chopped vegetables and 100 mL of acetone are placed in a blender cup and shaken vigorously on a mechanical shaker for 30 min. The homogenate is filtered under vacuum through a funnel fitted with a filter paper, and the residue is rehomogenized with 50 mL of acetone and then filtered again. The filtrates are combined and concentrated to about 20 mL using a vacuum rotary evaporator below 40 °C.

(2) Brown rice, wheat and soybean

Cereal samples are milled with an ultracentrifuge mill and sieved through a 42-mesh screen, then 20 g of the homogenized sample are transferred into a 300-mL Erlenmeyer flask and soaked in 40 mL of distilled water for 60 min. After 100 mL of acetone have been added to this flask, the same procedure as described in the case of vegetables is carried out.

Acifluorfen (an acidic diphenyl ether herbicide) is often difficult to extract from the complex soybean matrix. Therefore, Nemoto and Lehotay[4] developed the method of pressurized liquid extraction [(PLE), Hydromatrix (diatomaceous earth material) as a sample dispersant ingredient is used] followed by capillary electrophoresis (CE). PLE was performed with an ASE 200 instrument (Dionex, Sunnyvale, CA, USA), with the operating conditions of 2000 psi, 100 °C, a 5-min extraction time, 100% solvent flush of the vessel (one cycle), and a 1-min purge with nitrogen. Approximately 24 mL of acetonitrile–0.05 N HCl (7 : 3, v/v; pH 2) were added to 3 g of soybean sample which was ground using a centrifugal mill to pass through a 60-mesh screen and 1.5 g of Hydromatrix. The mixed solution was extracted by PLE.

2.2.2 Cleanup

(1) Liquid–liquid partition

(a) NaCl solution–n-hexane

A 100–200-mL volume of 5% NaCl aqueous solution and 100 mL of n-hexane are added to the extracts prepared in Section 2.2.1, and the mixed solution is shaken vigorously for 5 min. The n-hexane layer is separated and a further 50 mL of n-hexane are added to the aqueous layer (lower layer) and shaken again. The n-hexane layers are collected, dehydrated with ca 20 g of anhydrous Na_2SO_4, and concentrated using a vacuum rotary evaporator below 40 °C, and the residue is dried under a gentle stream of pure nitrogen and dissolved in ca 20 mL of n-hexane.

(b) Acetonitrile–n-hexane

The acetonitrile–n-hexane partitioning is an additional procedure in the residue analysis of plant samples having high oil content (e.g., rice grain, bean, and corn). A 30-mL volume of acetonitrile is added to the above-mentioned n-hexane layer of plant extract and the mixed solution is shaken vigorously. The acetonitrile layer is separated, a further 30 mL of acetonitrile are added to the n-hexane layer, and the mixed solution is shaken vigorously. The combined acetonitrile layers are carefully concentrated to dryness.

(c) Microporus diatomaceous column (MDC)

The MDC is an effective procedure for the cleanup of the sample. The sample residue obtained in Section 2.2.1 is transferred to the MDC (Chem Elut, etc.) and the column is left at room temperature for 10 min. Bifenox is eluted with 80 mL of n-hexane, and the eluate from the MDC is concentrated to near dryness below 40 °C.[5]

(2) Column chromatography

(a) SPE silica gel cartridge cleanup

An SPE silica gel cartridge is prewashed with 10 mL of n-hexane to remove any contaminants from the cartridge. The sample residue in the flask is loaded on the cartridge with 6 mL of acetone–n-hexane (1 : 49, v/v) and the eluate is discarded. Bifenox is eluted with 8 mL of acetone–n-hexane (1 : 9, v/v) and the eluate is concentrated to near dryness below 40 °C, then dissolved with acetone to an appropriate volume for analysis by gas chromatography (GC).[6] Several types of SPE cartridge such as C_{18} (octadecylsilane), alumina-N (alumina-neutral), silica, NH_2 (aminopropyl) and SAX (anion exchange) were evaluated for the cleanup of acifluorfen from soybean extracts. SPE using SAX seems not to be appropriate among the five cartridges to obtain a high recovery. For example, SPE using the C_{18} cartridge recovered >90% of acifluorfen when 10 mL of 0.05 N HCl, 10 mL of water, and 7 mL of acetone were loaded sequentially. The procedure using the C_{18} cartridge was able to remove salts from the soybean extracts effectively.[4]

(b) Silica gel column chromatography

In the case of silica gel column chromatography, 10 g of Kieselgel 60 suspended in adequate amounts of diethyl ether–n-hexane (1 : 19, v/v) are placed in a chromatographic tube plugged with cotton wool at the bottom. On the top of the silica gel column, 10 g of anhydrous Na_2SO_4 are placed and the solvent is drained. A 5-mL volume of the n-hexane solution prepared by partitioning between NaCl solution and n-hexane [(1) (c)] is concentrated to dryness and the residue is transferred completely to a column with two portions of 5 mL of diethyl ether–n-hexane (1 : 19, v/v). Bifenox is eluted with 100 mL of diethyl ether–n-hexane (3 : 17, v/v), the eluate is concentrated using a rotary evaporator, and acetone is added to the concentrated residue for GC analysis.

(c) Florisil column chromatography

In place of silica gel, Florisil is also used as the adsorbent in column chromatography. Purification of chlornitrofen using a Florisil column is as follows: after installing a column packed with 10 g of Florisil suspended in n-hexane, the sample solution is added continuously to the column and the initial eluate is discarded. A 100-mL volume of diethyl ether–n-hexane (1 : 19, v/v) is charged to the Florisil column and the eluate is discarded. Chlornitrofen is eluted with 30 mL of this mixture and the eluate is concentrated to dryness before the addition of acetone for GC analysis.[6]

Examination of the elution solvent from the Florisil column to purify bifenox and chlomethoxyfen was carried out. Bifenox is eluted with 100 mL of acetone–n-hexane (1 : 9, v/v) after discarding 100 mL of diethyl ether–n-hexane (3 : 7, v/v) eluate, and chlometoxyfen is also eluted with 100 mL of acetone–n-hexane (5 : 95, v/v) after discarding 100 mL of n-hexane eluate.[7]

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(3) Extraction and cleanup of diphenyl ether herbicide metabolites in plants

The purification of chlornitrofen and the reduced metabolite, 2,4,6-trichlorophenyl 4-aminophenyl ether (CNP-NH_2) in brown rice and vegetables was investigated.[8]

A 20-g amount of the milled brown rice or minced vegetable is transferred into a 300-mL Erlenmeyer flask. After 100 mL of 0.2 M KOH–acetone (1 : 9, v/v) have been added to the flask, the mixture is shaken vigorously on a mechanical shaker for 30 min. The homogenate is filtered under vacuum through a funnel fitted with a filter paper, and the residue is rehomogenized with 70 mL of the same solution and filtered again. The filtrates are combined and concentrated to about 40 mL using a vacuum rotary evaporator below 40 °C.

The extracts are transferred to a flask which contains 100 mL of 2% Na_2SO_4 in 0.1 M KOH aqueous solution and 100 mL of n-hexane, and the flask is shaken vigorously for 5 min. The n-hexane layer is separated, a further 50 mL of hexane are added to the aqueous layer and the mixed solution is shaken. The combined n-hexane layers are transferred into a separatory funnel containing 100 mL of 0.2 M HCl and shaken vigorously on a mechanical shaker for 5 min. The two layers are separated for the determination of chlornitrofen in the n-hexane layer and CNP-NH_2 in the aqueous layer.

The n-hexane layer is dried with ca 50 g of anhydrous Na_2SO_4, filtered through a funnel fitted with a filter paper, concentrated to about 1 mL under vacuum below

40 °C and dried under a gentle stream of pure nitrogen. The residue is dissolved in 5 mL of n-hexane and loaded on the column suspended with 10 g of Florisil in an adequate volume of n-hexane with ca 10 g of anhydrous Na_2SO_4 on the top of the Florisil. An additional 5 mL of n-hexane are transferred to the column, which is drained. Chlornitrofen is eluted with 70 mL of diethyl ether–n-hexane (3 : 17, v/v). The eluate is concentrated to about 1 mL under vacuum below 40 °C, dried under a gentle stream of pure nitrogen and dissolved in an appropriate amount of n-hexane for GC/ECD.

The aqueous layer (CNP-NH_2 layer) collected by liquid–liquid partitioning is transferred into a separatory funnel containing 20 mL of 4 M KOH aqueous solution and 100 mL of n-hexane, and shaken vigorously for 5 min. The n-hexane layer is collected. A further 100 mL of n-hexane are added to the aqueous layer and shaken again. The n-hexane layers are collected, dried with ca 50 g of anhydrous Na_2SO_4, concentrated to dryness carefully, and n-hexane solution of the residue is prepared for GC analysis.

2.2.3 Determination

(1) Gas chromatography

To determine the diphenyl ether herbicides in the samples, GC/ECD or GC/NPD is used in general. GC/ECD is preferred to GC/NPD owing to its higher sensitivity. An aliquot of GC-ready sample solution is injected into the gas chromatograph under the conditions specified below. In addition, multi- and confirmatory analysis of residues is carried out using gas chromatography/mass spectrometry (GC/MS) in the selected-ion monitoring (SIM) mode.

(a) GC/ECD

Bifenox: column, SPB-5 (15 m × 0.53-mm i.d., 1.0-μm film thickness); column, inlet and detector temperature, 250, 250 and 280 °C, respectively; gas flow rates, He carrier gas 20 mL min^{-1}, N_2 makeup gas 40 mL min^{-1}; injection volume, 2-μL. The retention time for bifenox is about 3 min.[6]

Chlornitrofen and CNP-NH_2: column, DB-1 (10 m × 0.53-mm i.d., 1.0-μm film thickness); column, inlet and detector temperature, 200, 250 and 280 °C, respectively; N_2 gas flow pressure, 1.6 kg m^{-2}; injection volume, 2 μL. The retention times for chlornitrofen and CNP-NH_2 is about 5 and 3.5 min, respectively.[8]

Simultaneous determination of three diphenyl ethers: column, 5% DC-200 (0.5–1 m × 2–3-mm i.d.); temperature, column 210–230 °C, inlet and detector 260 °C; gas flow rates, N_2 carrier gas 30–50 mL min^{-1}; injection volume, 2 μL. The retention times are approximately 4.5 min for bifenox, 2.5 min for chlornitrofen and 3.4 min for chlomethoxfen.[7]

(b) GC/NPD

Multiresidue analysis of 72 pesticides including three diphenyl ethers was carried out by GC/NPD under the following conditions: column, 5% DB-5 (30 m × 0.53-mm i.d., 0.53-μm film thickness); temperature, column 100 °C (1 min) increased at 5 °C min^{-1} to 280 °C (10 min), inlet and detector 280 °C; gas flow rates, He 11.2 mL min^{-1}, H_2 3.5 mL min^{-1}, air 110 mL min^{-1}; injection volume, 2 μL. The retention times

are approximately 35.5 min for bifenox, 34.6 min for chlornitrofen and 32.7 min for chlomethoxyfen.

Oxyfluorfen: column, fused-silica capillary column coated with cross-linked methyl silicone (25 m × 0.3-mm i.d., 0.52-μm film thickness); temperature, column 200 °C (1 min), 10 °C min^{-1} to 250 °C (5 min), inlet and detector 250 and 300 °C, respectively; gas flow rates, N_2 carrier gas 30 mL min^{-1}, N_2 makeup gas 30 mL min^{-1}, H_2 3.5 mL min^{-1}, air 110 mL min^{-1}; injection volume, 2 μL.[9]

(c) GC/MS

Chlornitrofen and nitrofen: conditions for GC/MS: column, cross-linked methyl silicone capillary (12 m × 0.22-mm i.d., 0.33-μm film thickness); column temperature, 60 °C (1 min), 18 °C min^{-1} to 265 °C; inlet, transfer line and ion source temperature, 260, 200 and 200 °C, respectively; He gas column head pressure, 7.5 psi; injection method, splitless mode; solvent delay, 3 min; electron ionization voltage, 70 eV; scan rate, 0.62 s per scan cycle; scanned mass range, m/z 100–400. The retention times for chlornitrofen and nitrofen were 11.8 and 11.3 min, respectively. The main ions of the mass spectrum of chlornitrofen were at m/z 317, 319 and 236. Nitrofen presented a fragmentation pattern with the main ions at m/z 283, 202 and 285.[10]

(2) HPLC

Okumura *et al.*[3] reported State regulatory programs for pesticide residues in food crops analyzed by the CDFA. In the multiresidue analysis of several organochlorine pesticides including diphenyl ether herbicides, bifenox, nitrofen and oxyfluorfen, HPLC has also been used.

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Bifenox, nitrofen and oxyfluorfen: HPLC conditions with post-column fluorescence reactor system: column, C-18 reversed-phase (25 cm × 4.6-mm i.d.); temperature, 40 °C; flow rate, 1 mL min^{-1}; flow composition, acetonitrile–water (1 : 4, v/v) (2 min), with increase in acetonitrile at 5% min^{-1} to 90% acetonitrile to acetonitrile–water (9 : 1, v/v) (2 min).

(3) CE

The determination of acifluorfen in soybean was performed using CE,[4] under the following conditions: capillary column (total length 83 cm, 65 cm to the detector, with a 3-mm pathlength, 75-μm i.d.); absorbance detector, 240 nm; capillary oven temperature, 20 °C; running buffer, 50 mM ammonium acetate buffer (pH, 4.75); applied voltage, 17 kV; injection, 0.4 min at 4 mbar; migration time, 20 min.

2.2.4 Evaluation

Quantitative analysis is performed by the calibration technique. A new calibration curve with a standard solution of each diphenyl ether herbicides is constructed, plotting the peak area against the amount of standard solution injected. Each diphenyl ether herbicide in the sample is measured by using the peak area for each standard. Before each set of measurements, the GC and HPLC system is checked by injecting more than one standard solution containing ca 0.01–2 mg L^{-1} of each compound.

2.2.5 Recoveries, limit of detection and limit of quantitation

The MDCs are estimated from an S/N of the diphenyl ether peaks of at least of 3 in the recovery test. With fortification levels between 0.2 and 0.5 mg kg^{-1}, recoveries of bifenox from brown rice matrices ranged from 85 to 102% with the limit of detection (LOD) and limit of quantitation (LOQ) being 0.010 mg kg^{-1} according to the analytical method of the Notification of the Ministry of the Environment, Japan. By the residue analysis method described in Section 2.2.2(3), recoveries of chlornitrofen and CNP-NH_2 from brown rice and vegetables with fortification levels of 0.04–0.10 mg kg^{-1} ranged from 82 to 98%. The LOD for each sample was 0.005 mg kg^{-1} for chlornitrofen and CNP-NH_2.[8]

The recoveries of nitrofen and oxyflourfen from green bean, bell pepper, lettuce and carrot fortified with 0.25 mg kg^{-1} were obtained using GC/MS. The average recoveries ranged from 106 to 127% and the LOD was 0.05 mg kg^{-1}.[10]

In the HPLC method for the regulatory system of the CDFA, the MDCs are 0.1 mg kg^{-1} for each of bifenox, nitrofen and oxyfluorfen.[3] In the determination of acifluorfen residues in soybeans using PLE and CE, the recovery of acifluorfen fortified with 0.1 mg kg^{-1} was between 70 and 72%.[4]

2.2.6 Calculation of residues

The residual amount (R, mg kg^{-1}) of diphenyl ether herbicides in the sample is calculated by the following equation:

$$R = (W_i / V_i) \times (V_f / G)$$

where

G = sample weight (g)
V_i = injection volume into gas chromatograph (μL)
V_f = final sample volume (mL)
W_i = amount of diphenyl ether herbicide for V_i read from the calibration curve (ng)

3 Analytical methodology for soil

3.1 Nature of the residues

The degradation of diphenyl ether herbicides in treated soil is rapid and mainly facilitated by soil microorganisms. Diphenyl ethers herbicides degrade much faster under flooded paddy field conditions than under upland conditions, and the value of the half-life (DT_{50}) in anaerobic soil conditions is about 4 days for bifenox and nitrofen and 6–7 days for chlornitrofen.[11] Water *et al.*[12] investigated the persistence of fluorodifen under aerobic soil conditions, and estimated that the DT_{50} was between 1.5 and 3.5 months for sandy loam and clay soils, respectively. It was reported that under Brazilian Savanna conditions, the dissipation time of fomesafen (DT_{50} = 38 days) was longer than that of acifluorfen (DT_{50} = 28 days).[13]

In flooded soils, the main metabolites of diphenyl ether herbicides are *p*-amino derivatives, adhering tightly to soil particles. The soil-bound residue in soil could be extracted with an organic solvent at 80–100 °C under alkaline conditions in >4 h. It was reported that at the earlier stages of degradation of chlornitrofen, nitrofen and chlomethoxyfen, the reduction rate of these herbicides increased with increase in the ferrous ion concentration of the system, and decreased with the redox potential of the soil.[14] From May to July, chlornitrofen was applied at the rate between 2.7 and 3.6 kg ha^{-1} to paddy fields in Hokkaido, Japan, and in the following spring the residues of chlornitrofen and CNP-NH_2 were found at levels of 0.18–1.33 and 1.16–3.36 kg ha^{-1}, respectively.[15] In nonflooded soils, however, the reduction of the metabolites of diphenyl ether herbicides is difficult to achieve.

The principal degradation products of bifenox are the free acid, 5-(2,4-dichlorophenoxy)-2-nitrobenzoic acid, and the amino derivatives, methyl 5-(2,4-dichlorophenoxy)anthranilate and its free acid, in flooded soil. A free acid is observed in nonflooded soil.[16] When [^{14}C]chlomethoxfen was used to treat rice field soil, chlomethoxfen was extensively transformed into unextractable products with organic solvents; however, the amine, the *N*-demethylated compound and the formylamino and acetylamino compounds were isolated and identified as the metabolites of chlomethoxfen.[17]

3.2 Analytical method

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Air-dried soil samples were screened through a 2-mm sieve, and the water content in the soil was calculated after holding at 105 °C for 5 h.

Diphenyl ether herbicides are generally extracted from 10 to 50 g of air-dried soil with an organic solvent such as acetone, methanol and benzene by sonication, mechanical shaking or Soxhlet extraction. If necessary, the extracts are then cleaned by column chromatography or SPE. The extract is evaporated completely to dryness and the residue is dissolved in an appropriate volume of the solvent for GC analysis. The reduced amine metabolites are extracted under alkaline conditions.

3.2.1 Extraction and cleanup

A 100-mL volume of benzene is added to the 20 g of air-dried soil and the mixture is shaken vigorously for 2 h. After extraction twice with 100 mL of benzene, the combined extract is filtered through filter paper and the filter cake is washed with an additional 20 mL of benzene. The benzene extracts are dried over anhydrous Na_2SO_4 and concentrated to dryness using a vacuum rotary evaporator. The residue is dissolved in an appropriate volume followed by GC/ECD analysis. For the monitoring of pesticide residues in soil, methanol for bifenox and oxyfluorfen and acetonitrile for nitrofen were recommended as the solvents for efficient extraction.[18]

Florisil column chromatography is effective in eliminating interfering substances in soil. The organic solvent extracts from soil samples are charged to a column plugged with Florisil which has been activated at 130 °C overnight before use. The effluents from the column with a mixed solvent such as n-hexane–acetone are concentrated to

dryness and the residue is dissolved in an appropriate amount of n-hexane for GC analysis.

The extraction efficiency of the SPE procedure for oxyfluorfen in soil was compared with that of Soxhlet extraction.[19] Ten grams of soil including the natural water contents were added to 5 mL of water and the mixture was shaken vigorously for 1 h. After extraction with 15 mL of methanol by sonication at 60 °C for 15 min, the mixture was subsequently shaken for a further 15 min by a mechanical shaker at room temperature. The extracts were transferred into an extraction reservoir containing 1 L of water and acidified to pH < 3 with 6 N HCl and then passed through an SPE (C_{18}) disk at a flow rate of about 50 mL min^{-1}. The absorbed oxyfluorfen was eluted with 2 × 5 mL of ethyl acetate and the eluate was concentrated and analyzed by gas chromatography/ion trap mass spectrometry (GC/ITDMS). In the case of Soxhlet extraction, 10 g of soil were extracted with 200 mL of n-hexane–acetone (1 : 1, v/v) for 24 h. The extract was dried through an anhydrous Na_2SO_4 column and concentrated for GC/ITDMS analysis. With a fortification level of 0.2 mg kg^{-1}, the mean recovery of oxyfluorfen was 80% for SPE and 97% for Soxhlet extraction.

3.2.2 Determination and evaluation

The residue levels of 46 pesticides, including oxyfluorfen in soil, were determined using GC/ITDMS as described in Section 3.2.1. The conditions for GC/ITDMS were as follows: column, fused-silica capillary (30 m × 0.25-mm-i.d.) with a 0.25-μm bonded phase of DB-5; column temperature, 50 °C (1 min), 30 °C min^{-1} to 130 °C, 5 °C min^{-1} to 270 °C; inlet and transfer temperature, 270 and 220 °C, respectively; He gas with column head pressure, 12 psi; injection method, splitless mode. The retention time and quantitation ion of oxyfluorfen were 23.9 min and m/z 252, respectively.[19]

3.2.3 Recoveries, limit of detection and limit of quantitation

In analyses at fortification levels of 1 and 10 mg kg^{-1} of chlornitrofen, nitrofen and chlomethoxyfen in soil, the recoveries varied from 96 to 103% for GC/ECD (2 m × 3-mm i.d. spiral glass column packed with 1.5% silicone GE SE-30; temperature of column, injector and detector, 220, 230 and 220 °C, respectively); the LOD was 0.1 mg kg^{-1}.[17] In the method reported by Bao *et al.*[19] using a combination of disk SPE with GC/ITDMS, the recovery of oxyfluorfen at fortification levels ranging from 0.01 to 0.4 mg kg^{-1} was between 100 and 102%; the LOD was 0.004 mg kg^{-1}.

3.2.4 Calculation of residues

Calculation of residues in soil was carried out as described in Section 2.2.6.

3.3 Analytical method for the metabolites of diphenyl ether herbicides in soil

Under flooded soil conditions, the diphenyl ether herbicides are substantially transformed into the amino derivatives, which are incorporated tightly into the soil particles. An analytical method for these amino derivatives in soil has been developed.

For the simultaneous residue determination of chlornitrofen and CNP-NH_2, 10 g of soil are placed in a flask containing 5 g of $Na_2SO_4 \cdot 9H_2O$ and 20 mL of 10 M NaOH aqueous solution and the mixture is refluxed overnight at 80 °C. After cooling, 30 mL of water, 5 g of diatomaceous earth, 2 g of copper and 100 mL of acetone are added and the mixture is shaken for 30 min. A further 50 mL of n-hexane are added to the aqueous layer and the mixed solution is shaken again. The combined n-hexane layers are filtered through a funnel fitted with a filter paper. The n-hexane extract is concentrated to ca 50 mL under reduced pressure. A 200-mL volume of 5% NaCl aqueous solution and 50 mL of n-hexane are added to the concentrates and shaken vigorously for 5 min. A further 50 mL of n-hexane are added and shaken again. The n-hexane layer is transferred to a separatory funnel. The cleanup procedure and residue determination are carried out as described in Section 2.2.3.

Niki and Kuwatsuka[17] reported a method involving trifluoroacetylation of the amino derivatives of chlornitrofen, nitrofen and chlomethoxyfen. A 1-mL volume of 10 M NaOH solution was added to 50 g of soil and the mixture was extracted with 100 mL of benzene. After separation and drying over anhydrous Na_2SO_4, the benzene solution was trifluoroacetylated by adding successively 1 mL of 0.1% trifluoroacetic anhydride in benzene and 1 mL of 0.1% triethylamine in benzene. The mixture was shaken for 30 s and diluted to 10 mL with benzene. To remove the excess of trifluoroacetic anhydride, about 2 mL of water were added to the mixture and shaken for 30 s. The benzene layer was dried over anhydrous Na_2SO_4 and injected for gas chromatography/flame ionization detection (GC/FID).

The GC/FID conditions were as follows: column, 1.5% OV-17 (2 m × 3-mm i.d.) glass column; N_2 carrier gas flow rate, 45 mL min^{-1}; temperature of injection port, column and detector, 240, 235 and 235 °C, respectively. The recoveries of these amino derivatives with fortification level ranging from 0.5 to 10.0 mg kg^{-1} were 62–101% for chlornitrofen, 62–101% for nitrofen and 58–101% for chlomethoxyfen, and satisfactory recoveries from soil were obtained at high concentrations, but the recoveries at lower concentration averaged about 66% for the least recovered compound. Interference from other substances in the soil extracts derived from the acetylation reaction was negligible.

DropBooks

4 Analytical methodology for water

4.1 Nature of the residues

Environmental pollution caused by pesticides has become a serious problem. Especially during and/or after pesticide application to crops, the pesticides are released into sensitive environmental areas, and also into ground and surface water, and could be harmful or dangerous to humans and other species. Therefore, very low concentrations of diphenyl ether herbicides in environmental waters must be monitored.

The concentration of chlornitrofen in river water released from flooded paddy fields 30–60 days after application was detected in the range 0.039–1.25 μg L^{-1}.[20] Further, it was reported that the DT_{50} of diphenyl ether herbicides in groundwater, river water and seawater were 17–84, 14–140 and 10–88 days for chlornitrofen, and 18–131, 4–206 and 6–23 days for bifenox, respectively. Diphenyl ether herbicides in

water are hydrolytically stable in the dark, but in light are rapidly degraded, and the DT_{50} of acifluofen and bifenox are ca 2 h and 24 min, respectively, at 250–400 nm.[2] In anaerobic conditions, chlornitrofen was decreased to below 5% of the dose by microorganisms by 7 days after application, and the metabolites identified were CNP-NH_2 and 4-aminophenyl 2,6-dichlorophenyl ether.[21]

4.2 Analytical method

Water samples of 500–1000 mL are extracted and purified simultaneously through an SPE cartridge such as Carbograph-1, C_{18} and RP-18, usually followed either by HPLC with ultraviolet (UV) or photoconductivity detection or by GC/ECD. The acidic-type diphenyl ether herbicides are derivatized with diazomethane and various kinds of chloroformates and determined by GC and HPLC.

4.2.1 Extraction and cleanup

In recent years, the extraction of diphenyl ether herbicides from water samples such as river water, groundwater and drinking water by SPE has increased in popularity.

Water samples (1000 mL of groundwater and drinking water, 500 mL of river water) are stirred artificially and drawn through a Carbograph-1 cartridge (LARA, Rome, Italy), which is fitted into a side-arm filter flask, at flow rates of 30–50 mL min^{-1}. The cartridge is then washed with 7 mL of water. Most of the water remaining in the cartridge is expelled under vacuum for ca 5 min and the residual water content is further decreased by slowly passing 1 mL of methanol through the cartridge. The neutral diphenyl ether herbicides (aclonifen, bifenox, fluoroglycofen, lactofen and oxyfluorfen) are then eluted with 8 mL of dichloromethane–methanol (4 : 1, v/v). Thereafter, to elute the acidic diphenyl ether herbicides (acifluorfen, bifenox acid and fomesafen), the cartridge is turned upside-down, and the herbicides are reverse-eluted by passing 10 mL of dichloromethane–methanol (4 : 1, v/v) acidified with formic acid through the cartridge at a flow rate of ca 5 mL min^{-1}. A 40-μL volume of 30% aqueous ammonia solution–methanol (1 : 1, v/v) is added to the eluate, and the latter is then concentrated to about 200 μL at 40 °C, under a gentle stream of nitrogen. The walls of the vials are washed sequentially with 100 μL of acetonitrile–water (1 : 1, v/v) and 100 μL of formic acid–acidified methanol (25 mM). The combined concentrate and washings containing neutral diphenyl ether herbicides are further concentrated to ca 200 μL and the final volume is carefully measured. The fraction containing the acidic diphenyl ether herbicides and metabolites is concentrated to dryness, and the residue is reconstituted with 250 μL of acetonitrile–methanol (1 : 1, v/v)–water (3 : 2, v/v) acidified with 100 mM formic acid. Volumes of 100 μL of both the solutions containing neutral and acidic diphenyl ether herbicides and the acidic compounds are injected on to the LC column.[22]

Chlornitrofen in river water (1000 mL) was determined using two Sep-Pak C_{18} cartridges connected together (Waters), which were rinsed with 5 mL of methanol, and cleaned and conditioned with 10 mL of water. Chlornitrofen was eluted from the cartridge with 10 mL of methanol after being rinsed with 3 mL of water–methanol

(7 : 3, v/v). The eluate was evaporated to dryness and the residue was dissolved in acetone, and injected into the GC/MS system.[23]

Acifluorfen, an acidic diphenyl ether herbicide, was extracted from 100 mL of the water samples after adjusting the pH to 1.0 with sulfuric acid and was eluted through 47-mm C_{18} and polystyrene–divinylbenzene (PS-DVB) resin extraction disks (Analytical International, PA, USA) with 20 mL of methanol–methyl *tert*-butyl ether (1 : 9, v/v). After drying the extract by passing it through a large Pasteur pipet containing 4 g of acidified anhydrous sodium sulfate, acifluorfen was esterified with diazomethane for analysis by GC.[24] Water samples were treated with diazomethane gas by a micromolar generation procedure. The methyl ester of acifluorfen was determined by GC/ECD. Butz and Stan[25] reported a simple method for the determination of acifluorfen residues in water. The water sample (100 mL) acidified to pH 1.5 was drawn through an RP-18 cartridge (Baker, Germany) at a flow rate of ca 8 mL min^{-1} and acifluorfen was eluted with 2 mL of methanol after drying the cartridge for 2–3 h under a gentle stream of nitrogen. Thereafter, the extract was treated with methyl, ethyl or butyl chloroformate to give the corresponding methyl, ethyl or butyl esters of acifluorfen and the esters were determined by GC/MS.

4.2.2 Determination and evaluation, recoveries, limit of detection, limit of quantitation and calculation of residues

The procedure for the determination, evaluation and calculation of residues of diethyl ether herbicides in water is carried out fundamentally by a similar procedure to the plant material method described in Sections 2.2.3 and 2.2.4.

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Lagana *et al*.[22] developed a new analytical method combining off-line SPE with liquid chromatography/ultraviolet/diode-array detection (LC/UV/DAD). The conditions for LC/UV/DAD were as follows: column, C_{18} packed Alltima (25 cm × 4.6-mm i.d.); precolumn, Supelguard, C_{18} (2 cm × 4.6-mm i.d.); absorbance, 290 nm by diode-array detection; mobile phase: for neutral diphenyl ether herbicides, acetonitrile–water (linear increase in the proportion of acetonitrile from 62 to 75% in 30 min, and to 100% in the following 5 min); for acidic diphenyl ether herbicides, phase (A) acetonitrile–methanol (1 : 1, v/v) acidified with 20 mM formic acid and phase (B) water acidified with 50 mM formic acid (linear increased in the proportion of phase A from 55 to 70% in 30 min, followed by 5 min at 100%); flow rate, 1 mL min^{-1} for both groups. The retention times for the five diphenyl ether herbicides, aclonifen, bifebox, fluoroglycofen, lactofen and oxyfluorfen were 12–24 min and for the three acidic diphenyl ether herbicides acifluorfen, bifenox acid and fomesafen they were 22–27 min. Recoveries and LODs from different natural drinking water and groundwater samples fortified at 50 μg L^{-1} and of river water fortified at 200 μg L^{-1} with aclonifen were 96–97% and 6.0–19.8 ng L^{-1} and 94–96% and 6–22 ng L^{-1} for bifenox, 90–91% and 7–10 ng L^{-1} for fluorglycofen, 91–96% and 6–32 ng L^{-1} for oxyfluorfen, 85–93% and 7–32 ng L^{-1} for lactofen, 96–100% and 5–26 ng L^{-1} for acifluorfen, 93–102% and 5–30 ng L^{-1} for bifenox acid and 94–97% and 4–18 ng L^{-1} for fomesafen. In groundwater and river water samples, higher detection limits were observed than in drinking water owing to the interference of matrix compounds.

Chlornitrofen in water samples was determined by GC/MS using an SPB-1 fused-silica capillary (15 m × 0.53-mm i.d., 0.5-μm film thickness), with the ionization

voltage and the ion-source temperature set at 70 eV and 150 °C, respectively.[23] Helium was used as the carrier gas at a flow rate of 30 mL min^{-1}. The temperatures of the column, separator and injection port were maintained at 170, 250 and 250 °C, respectively. The molecular ions of m/z 317, 236 and 173 of chlornitrofen were monitored. The recovery from the water samples at a fortification level of 2.5 μg L^{-1} was 79% and the LOD was 50 ng L^{-1}.

In the above-mentioned method by Hodgeson *et al.*,[24] acifluorfen was determined by GC/ECD after the extraction with a 47-mm PS-DVB disk and derivatization with diazomethane. The conditions for GC/ECD were as follows: column, DB-5 fused silica (30 m × 0.32-mm i.d., 0.25-μm film thickness); He carrier gas velocity, 25 cm s^{-1} (210 °C), detector makeup gas, methane–argon (5 : 95), 30 mL min^{-1}; column temperature, 50 °C (5 min), 10 °C min^{-1} to 210 °C (5 min) and to 230 °C (10 min); injection port and detector temperature, 220 and 300 °C, respectively; injection method, splitless mode. The recovery of acifluorfen from purified water, dechlorinated tap water and high humic content surface water fortified at 0.5–2.0 μg L^{-1} was 59–150% and the LOD was 25 ng L^{-1}. Acifluorfen after derivatization with various chloroformates was also determined by GC/MS using an SE-54 column (25 m × 0.20-mm i.d., 0.32-μm film thickness), and the average recovery of acifluorfen fortified between 0.05 and 0.2 μg L^{-1} was 78%.[25]

4.2.3 Enzyme-linked immunosorbent assay (ELISA) determination of acifluorfen and chlornitrofen

Miyake *et al.*[26] reported an ELISA method for the determination of pesticide residues in the aquatic environment. The polyclonal antibody and three monoclonal antibodies of acifluorfen were prepared by immunization of rabbits and mice with acifluorfen–bovine serum albumin conjugates. The polyclonal antibody reacted with acifluorfen at concentrations of 1.5–800 mg L^{-1}, while the monoclonal antibodies reacted with acifluorfen at concentrations of 1.5–144 mg L^{-1}. Among three monoclonal antibodies, AF 75-144 reacted with chlornitrofen, which did not react with the other two antibodies. It seems that the ELISA method is effective for the determination of herbicide residues in the aquatic environment.

References

1. T.R. Roberts, D.H. Hutson, P.W. Lee, P.H. Nicholls, and J.R. Plimmer, 'Metabolic Pathways of Agrochemicals,' Royal Society of Chemistry, Cambridge (1998).
2. C.D.S. Tomlin (ed.), 'The Pesticide Manual,' 12th edition, British Crop Protection Council, Farnham (2000).
3. D. Okumura, R. Melnicoe, T. Jackson, C. Drefs, K. Maddy, and J. Wells, *Rev. Environ. Contam. Toxicol.,* **118,** 87 (1991).
4. S. Nemoto and S.J. Lehotay, *J. Agric. Food Chem.,* **46,** 2190 (1998).
5. T. Iijima, M. Yanagi, E. Nagayoshi, and O. Matano, in 'Proceedings of the 19th Japan Pesticide Science Meeting,' Sapporo, p. 140 (1994) (in Japanese).
6. M. Takeda, Y. Ito, Y. Odanaka, K. Komatsu, Y. Maekawa, and O. Matano, 'Residue Analysis of Pesticides,' Cyuou Houki Syuppan, Tokyo (1995) (in Japanese).
7. S. Goto and S. Kato, 'Analytical Methods of Pesticide Residues: Addendum,' Soft Science, Tokyo (1987) (in Japanese).

8. M. Ueji, H. Kobayashi, and K. Nakamura, 'Analytical Methods of Pesticide Residues: 2002 Version,' Soft Science, Tokyo (2001) (in Japanese).
9. G. G. Ying and B.D. Williams, *Pestic. Manage. Sci.,* **56,** 277 (2000).
10. W. Liao, T. Joe, and W.G. Cusick, *J. Assoc. Off. Anal. Chem.,* **74,** 554 (1991).
11. T. Takematsu, 'Jyosouzai Kenkyu Souran,' Hakuyu, Tokyo (1985) (in Japanese).
12. J.P. Water, E.F. Eastin, and M.G. Merkle, *Weed Res.,* **10,** 165 (1970).
13. T. Cobucci, H.T. Prates, C.L.M. Falcao, and M.M.V. Rezende, *Weed Sci.,* **46,** 258 (1998).
14. M. Oyamada and S. Kuwatsuka, *J. Pestic. Sci.,* **14,** 321 (1989).
15. T. Yamada, *Weed Res. Jpn.,* **20,** 1 (1975).
16. M. Oyamada and S. Kuwatsuka, *J. Pestic. Sci.,* **3,** 401 (1978).
17. Y. Niki and S. Kuwatsuka, *Soil Sci. Plant Nutr.,* **22,** 223 (1976).
18. T.H. Dao, T.L. Lavy, and J. Dragun, *Residue Rev.,* **87,** 91 (1983).
19. M.L. Bao, F. Pantani, K. Barbieri, D. Burrini, and O. Griffini, *Int. J. Environ. Anal. Chem.,* **64**, 233 (1996).
20. J. Kanazawa, 'Chemistry of Pesticides in the Environment,' Godo Syuppan, Tokyo, p. 153 (1992) (in Japanese).
21. J. Kanazawa, *Environ. Monit. Assess.,* **9,** 57 (1987).
22. A. Lagana, G. Fago, L. Fasciani, A. Marino, and M. Mosso, *Anal. Chim. Acta,* **414,** 79 (2000).
23. H. Kobayashi, K. Ohyama, N. Tomiyama, Y. Jimbo, O. Matano, and S. Goto, *J. Chromatogr.,* **643,** 197 (1993).
24. J. Hodgeson, J. Collins, and W. Bashe, *J. Chromatogr.,* **659,** 395 (1994).
25. S. Butz and H.-J. Stan, *J. Chromatogr.,* **643,** 227 (1993).
26. S. Miyake, H. Hayashi, A. Kita, and H. Ohkawa, *Pestic. Sci.,* **51,** 49 (1997).

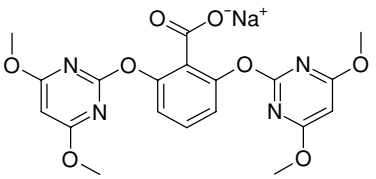
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Individual compounds

Bispyribac-sodium

Materials to be analyzed	Rice (grain, straw), soil, water
Instrumentation	Gas-chromatographic determination

1 Introduction

Chemical name (IUPAC)	Sodium 2,6-bis[(4,6-dimethoxypyrimidin-2-yl)oxy]-benzoate
Structural formula	
CAS No.	125401-92-5
Empirical formula	$C_{19}H_{17}N_4NaO_8$
Molar mass	452.3
Melting point	223–224 °C
Vapor pressure	5.04×10^{-9} Pa (25 °C)
Solubility	Water 73.3 g L^{-1} (25 °C), methanol 26.3 g L^{-1} (25 °C), acetone 0.043 g L^{-1} (25 °C)
Stability	Stable in water and under light
Use pattern	Bispyribac-sodium is a systemic post-emergence herbicide, used to control a broad range of weeds in rice
Regulatory position	The residue definition is for the parent, bispyribac-sodium, only

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2 Outline of method

Bispyribac-sodium is recovered as the free acid, bispyribac, from plant material and soil by acetonitrile–water (4 : 1, v/v) solvent extraction. After filtration, the acetonitirile is evaporated under reduced pressure. The aqueous residue is dissolved in buffer solution (pH 7.4) and washed with ethyl acetate to separate the impurities from the extract. Then the solution is acidified and extracted with ethyl acetate. The ethyl acetate is evaporated. The residue is methylated with trimethylsilyldiazomethane.

Handbook of Residue Analytical Methods for Agrochemicals.
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The methyl ester of bispyribac is cleaned up by silica gel column chromatography. Bispyribac-methyl is determined by gas chromatography with nitrogen–phosphorus detection (NPD).

For water, bispyribac is extracted with a C_{18} solid-phase extraction disk. After methylation, bispyribac-methyl is determined by gas chromatography.

3 Apparatus

Mill (coffee-mill type)
Grinder (cutting mills, Willey type)
Homogenizer (Polytron mixer)
Ultrasonic cleaner, UC-6100, 600 W, 28 kHz (Sharp)
Buchner funnel, 11-cm i.d.
Conical beaker, 500-mL
Round-bottom flasks, 1-L, 500-mL and 50-mL with ground joints
Rotary vacuum evaporator, 40 °C bath temperature
Separatory funnel, 300-mL
Funnel, 10-mm diameter
Chromatography column, glass, 15-mm i.d. × 400-mm with a stopcock
Silica gel column: Place a cotton-wool plug and then add anhydrous sodium sulfate in a layer 1-cm thick at the bottom of a glass chromatography column. Weigh 10 g of silica gel and pour it into the tube with n-hexane–ethyl acetate (5 : 1, v/v). Rinse the silica gel column with the same solvent system and place anhydrous sodium sulfate in a layer 1-cm thick on the top of the column
Gas chromatograph equipped with NPD
Microsyringe, 10-μL
Vacuum manifold (Waters)
Erlenmeyer flask, 300-mL
Bell jar
Two-piece filter funnel
Volumetric flasks, 5- and 10-mL

4 Reagents

Distilled water, high-performance liquid chromatography grade
Acetone, pesticide residue analysis grade
Acetonitrile, pesticide residue analysis grade
Ethyl acetate, pesticide residue analysis grade
n-Hexane, pesticide residue analysis grade
Diethyl ether, pesticide residue analysis grade
Bispyribac-sodium, analytical grade (Ihara Chemical Industry Co., Ltd)
0.1 M Phosphate buffer solution (pH 7.4), guaranteed reagent
0.2 M Acetate buffer solution (pH 4), guaranteed reagent
0.01 M Acetate buffer solution (pH 4): 0.2 M acetate buffer solution (pH 4) is diluted 20-fold
Sodium hydrogencarbonate, special grade

Anhydrous sodium sulfate, special grade
Sodium chloride, special grade
Filter aid, Celite 545 (Johns-Manville Products Corporation)
Filter paper, 110-mm diameter
Phosphoric acid, special grade
Silica gel, Wako gel C-200, residual agricultural chemical grade (Wako Pure Chemical Industries, Ltd)
Silica gel mini column, Sep-Pak Plus Silica: To set up, attach a Sep-Pak Plus Silica column to a vacuum manifold and rinse with 10 mL of ethyl acetate
Empore C_{18} extraction disks (3M): To set up, attach C_{18} extraction disks to a two-piece filter funnel and load 30 g of Filter Aid 400 on its surface. Wash the filter with 20 mL of acetonitrile and 20 mL of 0.01 M acetate buffer (pH 4) in that order
Filter Aid 400 (3M)
Trimethylsilyldiazomethane (Nakalai Tesque Inc.)
Bispyribac-sodium standard solution: Dissolve 100 mg of bispyribac-sodium in 100 mL of distilled water to prepare a 1000 mg L^{-1} solution
Bispyribac-methyl standard solutions: Transfer 100 μL of the bispyribac-sodium standard solution (1000 mg L^{-1}) into a 10-mL test-tube, evaporate the water and esterify bispyribac to its methyl ester according to the procedure described in Section 6.2.2. Dilute the methyl ester to prepare the standard solutions, equivalent to 0.2, 0.5, 1.0, 2.0 and 2.5 ng mL^{-1} of bispyribac-sodium
Cotton wool
pH test paper

DropBooks

5 Sampling and sample preparation

Soil samples are prepared by removing stones and plant materials and passing through a 5-mm sieve.

6 Procedure

6.1 Extraction

6.1.1 Rice straw and grain and soil samples

For a rice straw sample, weigh 10 g of the rice straw into a 500-mL conical beaker, add 200 mL of water–acetonitrile (1 : 4, v/v) and homogenize the mixture using a Polytoron for 2 min with ice–water cooling.

For a rice grain sample, weigh 20 g of the sample into a 500-mL conical beaker and add 100 mL of water–acetonitrile (1 : 4, v/v).

For a soil sample, weigh 30 g (dry soil) of the sample into a 300-mL Erlenmeyer flask and add 150 mL of water–acetonitrile (1 : 9, v/v). Sonicate the mixture for 30 min. Filter the extract through a filter paper overlaid with 20 g of Celite in a Buchner funnel into a 1-L round-bottom flask with suction. Rinse the beaker and the filter cake twice with 50 mL of acetonitrile. Combine the filtrates and concentrate to approximately

40 mL under reduced pressure at 35 °C to remove acetonitrile. Then the residue is processed as described in Section 6.2.1.

6.1.2 Water

To 100 mL of the water sample add 20 mL of 0.2 M acetate buffer (pH 4) to acidify the mixture to pH 4. Filter the solution through a C_{18} extraction disk with suction and rinse the disk with 50 mL of acetonitrile–water (1 : 19, v/v). Discard the eluate. Collect the bispyribac in a 500-mL round-bottom flask with 100 mL of acetonitrile. Evaporate the eluate to dryness under reduced pressure. Then the residue is processed as described below.

6.2 Cleanup

6.2.1 Liquid–liquid partition

Transfer the residue prepared as in Section 6.1.1 into a 300-mL separatory funnel with 25 mL of phosphate buffer solution (0.1 M, pH 7.4). Add 10 mL of saturated aqueous sodium chloride and 50 mL of 0.5 M sodium hydrogen carbonate to the funnel and shake the funnel vigorously for 1 min. Add 70 mL of ethyl acetate to wash the aqueous layer to the funnel, shake, separate, and discard the ethyl acetate layer. Repeat this extraction procedure three times. Add 2 mL of phosphoric acid and 20 mL of an acetate buffer solution (0.1 M, pH 4) to the aqueous layer and extract the mixture with 50 mL of ethyl acetate three times. Combine the extracts and filter into a 500-mL round-bottom flask through 60 g of anhydrous sodium sulfate supported by a plug of cotton wool in a funnel. Concentrate the filtrate to dryness under reduced pressure.

6.2.2 Methylation

Dissolve the residue prepared as in Section 6.1.2 or 6.2.1 in 1 mL of diethyl ether–methanol (4 : 1, v/v). Add 0.8 mL of 10% trimethylsilyldiazomethane in n-hexane to the mixture and allow the reaction mixture to stand at room temperature for 1 h. Concentrate the mixture to dryness under reduced pressure.

6.2.3 Column chromatography of bispyribac-methyl

Prepare a silica gel column as described in Section 3. Dissolve the residue prepared as in Section 6.2.2 in 3 mL n-hexane–ethyl acetate (5 : 1, v/v) and transfer the solution to the column. Rinse the flask twice with 5 mL of the same solvent system and transfer these solutions to the column. Allow the solution to percolate through the column and discard the eluate. Add 180 mL of n-hexane–ethyl acetate (5 : 1, v/v) to the column. Discard the first 80 mL eluate and collect the second 100 mL of eluate in a 300-mL round-bottom flask. Concentrate the eluate to dryness under reduced pressure.

6.3 Gas-chromatographic determination

Dissolve the residue prepared as in Section 6.2.3 in acetone. Transfer the solution into a volumetric flask, and make up to a given volume, e.g. 5 mL (V_{End}) with acetone. Inject an aliquot of the solution (V_i) into the gas chromatograph.

Operating conditions

Gas chromatograph	HP 5890A (Hewlett-Packard)
Column	DB-1301, 1.0-µm thickness, 30-m × 0.53-mm i.d. (J&W Scientific)
Column temperature	260 °C
Injection temperature	260 °C
Detector	Nitrogen–phosphorus detector
Temperature	270 °C
Gas flow rates	Helium carrier gas, 20 mL min^{-1} Helium make-up gas, 10 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 100 mL min^{-1}
Attenuation	2^{-1}
Chart speed	0.5 cm min^{-1}
Injection volume	1–5 µL
Retention time for bispyribac-methyl	5.8 min

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7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Prepare a calibration curve by injecting bispyribac-methyl standard solutions, equivalent to 0.2, 0.5, 1.0, 2.0 and 2.5 ng of bispyribac-sodium, into the gas chromatograph. Measure the heights of the peaks obtained. Plot the peak heights in millimeters against the amounts of bispyribac-sodium injected in nanograms. Measure the peak height of bispyribac-methyl on the chromatogram of the sample solution and quantify bispyribac-sodium by comparing the peak height with a calibration curve.

7.2 Recoveries, limit of detection and limit of determination

Untreated control samples of rice grain, rice straw, soil, and water were fortified with bispyribac-sodium. Fortification levels were 0.01–0.2 mg kg^{-1} for plant materials, 0.1 mg kg^{-1} for soil, and 0.002 mg L^{-1} for water. The following recoveries were obtained: 73–84% from rice grain, 75–85% from rice straw, 98–102% from soil, and 93% from water. The routine limits of detection were 0.005 mg kg^{-1} for rice grain, 0.01 mg kg^{-1} for rice straw, 0.005 mg kg^{-1} for soil, and 0.05 µg L^{-1} for water.

7.3 Calculation of residues

The residue R, expressed in mg kg^{-1} bispyribac-sodium, is calculated from the following equation:

$$R = \frac{W_A \times V_{End}}{V_i \times G}$$

where

G = sample weight (g)
V_{End} = final volume of sample solution from Section 6.3 (mL),
V_i = portion of volume V_{End} injected into the gas chromatograph (μL)
W_A = amount of bispyribac-sodium for V_i read from the calibration curve (ng).

8 Important points

Methylation of the residues of the ethyl acetate extract prepared in Section 6.2.1 should immediately be performed with trimethylsilyldiazomethane, because bispyribac is unstable under acidic conditions.

The extracts of plant, soil and water samples, if necessary, should be purified with the following method prior to methylation: Dissolve the residue prepared as in Section 6.1.2 or 6.2.1 in 5 mL of ethyl acetate and transfer the solution into a silica gel mini column. Rinse the column with 15 mL of ethyl acetate. Allow the solution to percolate through the column and discard the eluate. Collect the bispyribac in a 50-mL round-bottom flask with 20 mL of methanol–ethyl acetate (3 : 7, v/v). Evaporate the eluate to dryness under pressure.

Bispyribac in water samples can be directly quantified by high-performance liquid chromatography (HPLC) using an ultravilot (UV) detector without methylation.

Operating conditions

High-performance liquid chromatograph	HP1100 (Hewlett-Packard)
Column	CAPCELL PAK C_{18} UG120 (250 mm × 4.6-mm i.d.) (Shiseido)
Detector	UV 246 nm
Mobile phase	Acetonitrile–water–phosphoric acid (50 : 50 : 0.02, v/v/v)
Flow rates	1.5 mL min^{-1}
Retention time	5.2 min

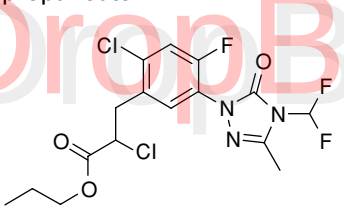
Bispyribac-sodium in rice grain (0.1 mg kg^{-1}), rice straw (0.2 mg kg^{-1}), and soil (0.1 mg kg^{-1}) stored at −20 °C was stable for 60 days.

Yoshihiro Saito, Mitsumasa Ikeda and Akira Yagi
Kumiai Chemical Industry Co., Ltd, Shizuoka, Japan

Carfentrazone-ethyl

Materials to be analyzed	Field corn grain, forage, stover and processed parts (grits, meal, flour, starch and oils); sweet corn ears, forage and stover; soybean seed and processed parts (meal, hulls and oil); wheat grain, forage, hay and straw; rice grain, straw and processed parts (hulls, bran and polished rice); sorghum grain, forage and stover; cotton seed, gin trash and processed parts (meal, hulls and oil); grape and raisins; and bovine milk, cream, liver, kidney, fat and muscle.
Instrumentation	Gas-chromatographic determination for plant and animal matrices.

1 Introduction

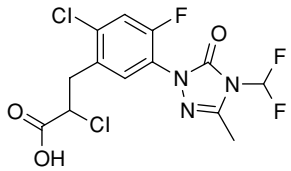
Chemical name (IUPAC)	Ethyl α,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoate
Structural formula	
Empirical formula	$C_{15}H_{15}N_3O_3F_3Cl$
Molar mass	412.2
Boiling point	350–355 °C
Physical state/odor	Viscous yellow/orange liquid with a very faint petroleum-like odor
Vapor pressure	1.2×10^{-7} mmHg (25 °C)
Water solubility	22 mg L^{-1} (25 °C)
Specific gravity	1.46 g mL^{-1} (20 °C)
Stability	Stable at pH 5, moderately stable at pH 7 and 9
Other properties	Undergoes hydrolysis rapidly. The half-life ($t_{1/2}$) of carfentrazone-ethyl in aqueous photolysis at pH 5 is 8.3 days of sunlight exposure.

DropBooks

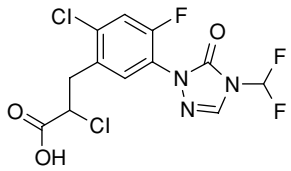
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Use pattern	Carfentrazone-ethyl is a rapid-acting, post-emergent contact herbicide that provides good control over broadleaf and sedge weeds in cereal grain crops. The product is also being developed for total vegetation control (TVC) as a potato desiccant and as a cotton defoliant. Currently, carfentrazone-ethyl is registered for agricultural use in the USA on soybeans and cereal grain crops and as a cotton defoliant, in Europe on small grain crops, and in Asia on wheat.
Regulatory position	The metabolism of carfentrazone-ethyl in animals and plants is similar. The major plant metabolites are carfentrazone-chloropropionic acid (C-Cl-PAc), 3-desmethylcarfentrazone-chloropropionic acid (DM-C-Cl-PAc), and 3-hydroxymethylcarfentrazone-chloropropionic acid (HM-C-Cl-PAc). The major animal metabolites are carfentrazone-chloropropionic acid (C-Cl-PAc) and carfentrazone-propionic acid (C-PAc). The tolerance expression for livestock and plant commodities is carfentrazone-ethyl plus the ester hydrolysis product, C-Cl-PAc.

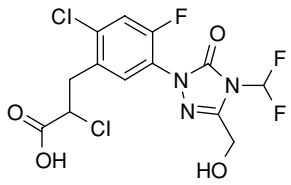
Carfentrazone-chloropropanoic acid (C-Cl-PAc)
α,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoic acid



3-Desmethylcarfentrazone-chloropropanoic acid (DM-C-Cl-PAc)
α,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluoro-benzenepropanoic acid



3-Hydroxymethylcarfentrazone-chloropropanoic acid (HM-C-Cl-PAc)
α,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-hydroxymethyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoic acid



2 Outline of method

The analytical method for carfentrazone-ethyl and its major metabolites in/on corn grain, grits, meal, flour, and starch (nonoil matrices) consists of extractions with acetone and deionized water, followed by a partition with hexane, which allowed the separation of the parent carfentrazone-ethyl from the acid metabolites. The hexane

fraction, containing the carfentrazone-ethyl, is cleaned up with a silica gel (SI) solid phase extraction (SPE) cartridge. The aqueous phase, containing the acid metabolites, is acidified (1 N HCl), boiled under reflux, partitioned with methylene chloride, derivatized using boron trifluoride in methanol (BF_3–MeOH) and acetic anhydride, and cleaned up with an SI SPE cartridge. The carfentrazone-ethyl is quantitated in a gas chromatograph equipped with a DB-17 Megabore capillary column and an electron capture detector. The acid metabolite derivatives are quantitated using a gas chromatograph equipped with a DB-5 narrow-bore capillary column and a mass-selective detector.

This enforcement method has been validated on the (raw agricultural commodities) (RAC) and processed parts of various crops. The method limit of quantitation (LOQ) was validated at 0.05 mg kg^{-1} and the method limit of detection (LOD) was set at 0.01 mg kg^{-1} for all of the crop matrices. The method flow chart is presented in Figure 1.

3 Apparatus

AccessChrom or TurboChrom data acquisition software, running on a MicroVax
Balance, Analytical PM 2000, Mettler
Balance, top loading, Mettler
Blender, Omni, equipped with a macro generator (20-mm diameter × 145-mm long w/sawteeth, part No. 15401, cat. No. 17105) or equivalent such as a Tekmar Tissuemizer
Boiling stones, Hengar
Buchner filter funnels, porcelain, 10.5-cm i.d., Coors
Capillary column, DB-35, 15 m × 0.25-mm i.d., 0.25-µm, J&W Scientific
Capillary column, DB-17, 30 m × 0.53-mm i.d., 1.0-µm, J&W Scientific
Centrifuge tubes, 15-mL, graduated, Pyrex, 0.1-mL
Centrifuge tubes, 50-mL, graduated, polypropylene, VWR (cat. No. 21008-714)
Condensers, Graham coil, Pyrex, 41 × 500-mm with T 24/40 joint
Cylinders, graduated, 10, 50, 100, 250-mL
Cylinders, mixing, 250-mL, graduated
Filtration tubes (6-mL capacity) containing a (20-µm pore size) polyethylene frit, VWR (cat. No. JT7121-6)
Filter paper, Whatman 934-AH, 7-cm diameter, VWR (cat. No. 28496-955)
Flasks, vacuum filter, Pyrex, 500-mL
Flasks, round-bottom boiling, Kontes, 50-mL, T 45/50 joint
Gas chromatograph, Hewlett-Packard (HP) 5890 equipped with an HP 7673A autosampler and an electron capture detector
Gas chromatograph, HP 5890 equipped with an HP 7673A autosampler and an HP 5972 mass-selective detector
Gas chromatograph injector liner [for gas chromatography/electron capture detection (GC/ECD)], cyclouniliner insert, Restek (cat. No. 20337)
Gas chromatograph injector liner [for gas chromatography/mass spectrometry (GC/MS)], cyclo-double gooseneck, 2 mm, Restek (cat. No. 20907)
Heating mantles, 500-mL, Glas-Col
Injection vials, 2-mL, Wheaton

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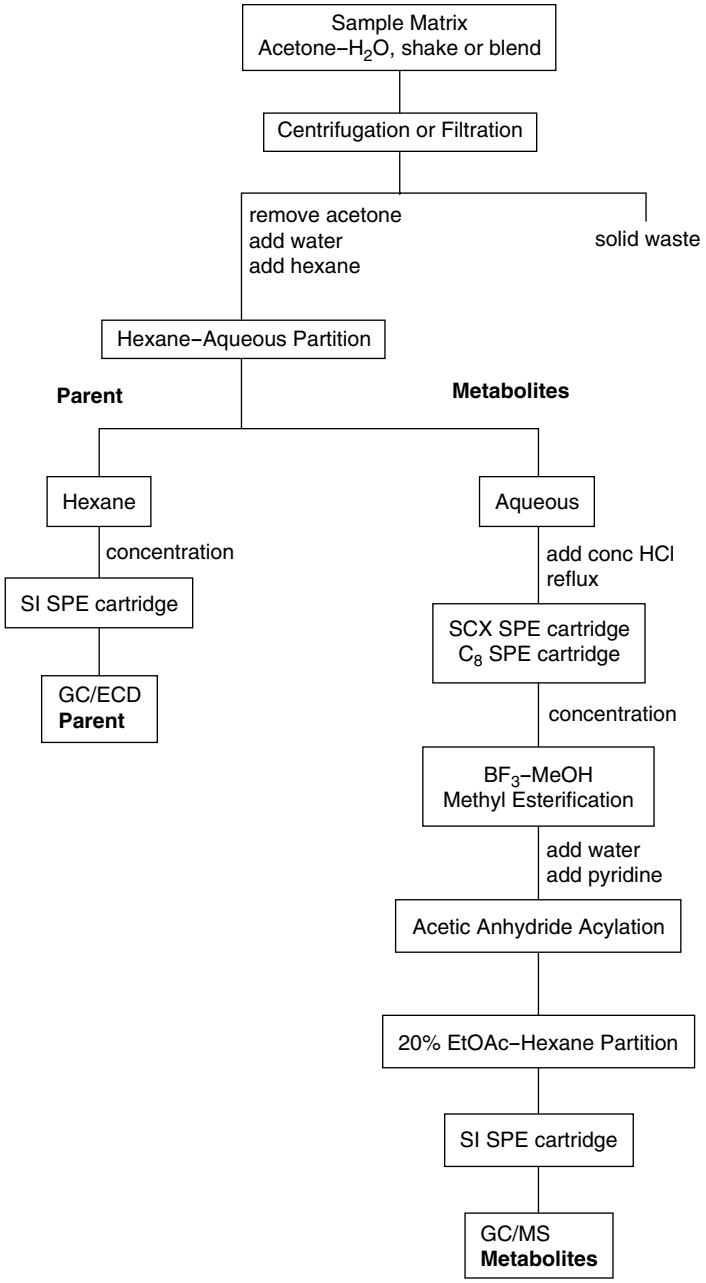


Figure 1 Method flow chart for carfentrazone-ethyl determination

Injection vial crimps, 11-mm, Teflon/silicone/Teflon, Sun Brokers
Microsyringes, (25-, 50-, 100-, 250-, 500-μL), Hamilton
Mill, Hobart
Mill, Wiley (Model ED-5)
N-EVAP evaporator, Organomation
Single-tube vortexer, VWR
Pipets, disposable (5.75- and 9-in lengths)
Pipets, volumetric pipette bulbs
Reducing adapters (SPE), plastic, Supelco
Reservoirs, plastic, 75-mL
Screw-capped glass tubes, 50 × 150-mm
SPE cartridge, C_{18} (1-g), Bakerbond, VWR (cat. No. JT7020-07)
SPE cartridge, SCX (1-g), Varian (part No. 1225-6011)
SPE cartridge, silica gel (1-g), J.T. Baker, VWR (cat. No. JT7086-07)
Test-tubes, glass, 25 × 150-mm
Stainless-steel blending cups, 400-mL capacity, Omni (cat. No. 17079)
TurboVap evaporator, Zymark
TurboVap centrifuge tube support rack, Zymark
TurboVap vessels, 200-mL, Zymark
TurboVap vessel support rack, Zymark
Visiprep vacuum manifold, Supelco
Visidry vacuum manifold drying attachment, Supelco

4 Reagents

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Acetic anhydride, ACS Reagent Grade, Sigma Chemical (product No. A6404) or Aldrich (product No. 11,004-3)
Acetone, Resi-Analyzed, J.T. Baker
Acetonitrile, HPLC grade, J.T. Baker
Boron trifluoride (14% in methanol), Sigma Chemical (product No. 13-1127)
Ethyl acetate, Pesticide Grade, J.T. Baker
Hexane, Resi-Analyzed, J.T. Baker
Hydrochloric acid (HCl, 36.5–38.0%), J.T. Baker
Hydrion pH buffer, VWR (cat. No. 34175-220)
Methanol, Resi-Analyzed, J.T. Baker
Methylene chloride, Resi-Analyzed, J.T. Baker
pH indicator strips (EM Science), VWR (cat. No. EM-9590-3)
Pyridine, Fisher (99.9%) or Sigma Chemical (product No. P-4036)
Sodium sulfate, anhydrous, J.T. Baker

Equivalent equipment and reagents may be substituted as appropriate, unless specified otherwise in the method.

5 Sampling and preparation

Prior to analysis, the samples were chopped and finely ground with liquid nitrogen using a large Hobart (forage, hay, fodder, straw and bovine tissue samples) or a Wiley

mill (grain and seed samples). Recently, frozen crop matrices were processed more effectively with Robot Coupe vertical cutter/mixer without liquid nitrogen.

6 Analytical procedures for nonoil crop matrices

6.1 Sample extraction, filtration and concentration

Weigh 2.5 or 5 g of crop matrix into a blending vessel. Fortify samples at this point with the appropriate analytical standards. Allow the solvent to evaporate. Add 100 mL of acetone–water (4 : 1, v/v) and blend the mixture using an Omni mixer equipped with a macro generator for 5 min at 6000–7000 rpm. Filter the sample through a Whatman 934 AH glass-fiber filter paper on a Buchner funnel/vacuum flask setup. Rinse the blending cup and filter cake with 100 mL of acetone. Transfer the filtrate into a 200-mL TurboVap vessel.

Concentrate the sample (remove acetone) under nitrogen to ca 20–25 mL using a TurboVap (water-bath at 50 °C). Transfer the sample into a 50-mL polypropylene centrifuge tube. Rinse the TurboVap vessel with 5 or 10 mL of pH 6 buffer solution. The amount of pH 6 buffer required depends on the matrix being analyzed and should be determined as needed. All matrices need 5 mL of the buffer solution to adjust the sample to pH 6, except for sweet corn (ears, forage, and stover), which requires 10 mL. Add the rinse buffer to the sample. Rinse the TurboVap vessel with 10 mL of hexane and add the hexane to the sample.

6.2 Partition

Vigorously mix the aqueous and hexane fraction to partition carfentrazone-ethyl into the hexane fraction. Centrifugation may be necessary to break any emulsion that occurs. Remove and collect the hexane fraction for analysis of carfentrazone-ethyl. Partition the aqueous fraction with an additional 10 mL of hexane and add the hexane to the hexane from the first partition step. The aqueous fraction will be used for the analysis of the acid metabolites (see below).

6.3 Determination of carfentrazone-ethyl

6.3.1 Cleanup

Concentrate the hexane fraction (20 mL) from the previous hexane–aqueous partition to 3 mL in a TurboVap at ca 50 °C.

For grain and forage matrices, condition a 1-g/6-mL SI SPE cartridge with 1 cartridge volume (1 CV), (1-g/6-mL) of hexane–ethyl acetate (9 : 1, v/v) followed by 1 CV of hexane (vacuum at 1 inHg). Load the 3-mL sample onto the cartridge, but do not elute the sample yet. Rinse the tube with 3 mL of hexane and also load this rinsate onto the cartridge. Drain the 6 mL of sample solution through the SI cartridge (vacuum at 1 inHg) and discard the eluate. Rinse the SI cartridge with 9 mL of hexane–ethyl acetate (9 : 1, v/v) and discard the rinsate. Elute and collect the sample with an additional 12 mL of hexane–ethyl acetate (9 : 1, v/v) (vacuum at 1 inHg).

For fodder, hay or straw matrices, in order to exclude an interference which only occurs in the dry matrices, a slightly less polar elution solvent (7.5% vs 10% ethyl acetate in hexane) and a larger volume (18 mL) are used.

Concentrate the sample to 0.1 mL in a TurboVap at ca 50 °C and adjust the sample to a final volume of 1.0 mL with acetonitrile. Note: there is the potential for loss of analyte if the samples go to dryness at this step. Analyze the sample for parent carfentrazone-ethyl by GC/ECD.

6.4 Determination of acid metabolites

6.4.1 Acid reflux

Transfer the aqueous fraction from the hexane–aqueous partition (25–30 mL) into a 50-mL round-bottom flask. Add 3–3.5 mL of concentrated HCl (such that the final acid concentration is ≥1 N and several boiling chips to the round-bottom flask and reflux the sample for 1 h under a water-cooled condenser. This acid reflux step will cleave any conjugated acid metabolites in the crop matrices.

6.4.2 SCX/C_{18} SPE cartridges

Allow the hydrolyzed sample to cool before handling. Assemble tandem SPE cartridges (SCX cartridge on top of the C_{18} cartridge) and install them on the vacuum manifold. Condition both the SCX (Varian, 1-g), and the C_{18} SPE cartridges (Bakerbond, 1-g) in series with methanol (1 CV) and then with 0.25 N HCl (1 CV) using 5 inHg of vacuum. After the 0.25 N HCl reaches the top of the column packing of the SCX cartridge, turn off the vacuum. Add an additional 0.5 CV of 0.25 N HCl and attach an SPE filtration cartridge with just a frit installed in the cartridge (no packing material) on top of the SCX cartridge. Attach a reducing adapter and a 75-mL reservoir to the top of the SPE cartridge containing the frit. Decant the hydrolyzed sample into the reservoir. Rinse the round-bottom flask with 40 mL of deionized water but do not add the rinsate to the hydrolyzed sample at this point. With the cartridge valve opened, apply a vacuum at 7–10 inHg and drain and discard the hydrolyzed sample. When the last of the hydrolyzed sample has passed through the SCX cartridge, add 40 mL of deionized water rinsate to the reservoir and drain the rinsate through all three cartridges. Discard the deionized water rinsate. Continue the vacuum of 7–10 inHg until all of the filtrate has eluted through all three cartridges.

Remove the reducing adaptor, reservoir, filtration cartridge, and the SCX cartridge and dry the C_{18} SPE cartridge with nitrogen for at least 60 min using a drying manifold. Elute and collect the analytes from the C_{18} SPE cartridge with 12 mL of dichloromethane–methanol (19 : 1, v/v). Concentrate the sample under nitrogen using the TurboVap to 0.1–0.25 mL (water-bath at 50 °C). Note: there is the potential for loss of analytes if the samples go to dryness at this step.

6.4.3 First derivatization (methyl esterification)

Add 1 mL of boron trifluoride in methanol (14% by weight) to the sample solution, vortex the solution and allow the sample to react for 45 min in a water-bath at 50 °C. After methylation, add 2 mL of water. If analysis of HM-C-Cl-PAc is not required,

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extract the methylated analytes with 5 mL of hexane and proceed to clean up on the SI SPE cartridge.

Partition the sample in methanol twice with 2 mL of dichloromethane (DCM), remove the DCM after each partition step and pass the sample in DCM through a 6-mL filtration tube containing a polyethylene frit and packed with 1 g of anhydrous sodium sulfate. The use of the anhydrous sodium sulfate can be eliminated if great care is taken when removing the DCM from each partition step so that no water is included with the DCM. If water droplets are present in the DCM fraction, carefully remove them with a small pipet. The DCM is then concentrated in a Turbovap to 0.1 mL at 50 °C. Note: there is the potential for loss of analytes if the samples go to dryness at this step.

6.4.4 Second derivatization (acylation)

Add 0.5 mL of acetic anhydride and 0.5 mL of pyridine to the sample solution, vortex the solution and allow the sample to react for 45 min in a water-bath at 50 °C. This procedure acylates the hydroxyl group on the HM-C-Cl-PAc-methyl ester.

After acylation, add 2 mL of water to the sample and partition the sample twice with 2 mL of hexane. Retain the 4-mL hexane fraction. The aqueous fraction containing excess acetic anhydride and pyridine is discarded.

6.4.5 Cleanup

Condition a 1-g/6-mL SI SPE cartridge with 1 CV of hexane–ethyl acetate (4 : 1, v/v) followed by 1 CV of hexane (vacuum at 1 inHg). Load the 4-mL sample onto the cartridge. Rinse the tube with 2 mL of hexane and also load the rinsate onto the cartridge. Drain the hexane containing the sample through the SI cartridge (vacuum at 1 inHg) and discard the eluate. Rinse the cartridge with 3 mL of hexane–ethyl acetate (4 : 1, v/v). Discard the rinsate. Elute and collect the sample with an additional 12 mL of hexane–ethyl acetate (4 : 1, v/v). Concentrate the sample under nitrogen to 0.5 mL in a TurboVap (water-bath at 50 °C), and adjust the sample to a final volume of 1.0 mL with hexane.

Analyze the sample by GC/MS, and monitor the ions at m/z 362 for C-Cl-Pac, 348 for DM-C-Cl-PAc, and 413 for HM-C-Cl-PAc.

6.5 Analytical procedures for crop refined oils

Crop refined oils should be dissolved in hexane and partitioned with deionized water in a separatory funnel. The hexane fraction containing the carfentrazone-ethyl should be further partitioned with acetonitrile, and the rest of the analytical procedures for the parent compound should be followed. Concentrated HCl is added to the aqueous fraction to make the solution 1 N and the samples are boiled under reflux for 1 h; the rest of the analytical procedures for the acid metabolites should be followed.

6.6 Analytical procedures for animal matrices

The analytical method to determine carfentrazone-ethyl and the major animal metabolites (C-Cl-PAc and C-Pac) in bovine matrices is similar to the method for crop matrices. The hexane–aqueous partition to separate carfentrazone-ethyl from the acid metabolites can be replaced by a C_{18} SPE cartridge. After the SPE, use 12 mL of water–acetonitrile (7 : 3, v/v) to elute the metabolites and then use 12 mL of hexane–ethyl acetate (4 : 1, v/v) to elute carfentrazone-ethyl after drying the cartridge. Follow the rest of the respective analytical procedures for carfentrazone-ethyl and the acid metabolites described in Sections 6.3 and 6.4. However, no reflux under boiling is necessary for the analysis of acid metabolites based on a goat metabolism study, because no conjugated acid metabolites were detected. Also, since HM-C-Cl-Pac is not analyzed for in the bovine matrices, no acylation is needed in the method. Analyze the metabolites by GC/MS, and monitor the ions at m/z 362 for C-Cl-Pac and 303 for C-PAc.

6.7 Instrumentation

Gas chromatography (GC) is used to analyze the sample extracts. Two detector systems are used, one for quantitation and the other for analyte confirmation and quantitation.

Operating conditions for carfentrazone-ethyl determination

Instrument	HP 5890 or 6890 gas chromatograph
Column	DB-17, phenyl/methyl (50 : 50) silicone gum, 30 m × 0.53-mm i.d., 1.0-μm film thickness
Inlet	Splitless injection mode
Detector	^{63}Ni electron capture
Temperatures	
Injection port	250 °C
Oven	150 °C/1 min (initial); 20 °C min^{-1} (ramp 1); 200 °C/0 min; 10 °C min^{-1} (ramp 2); 260 °C/10 min (final)
Detector	300 °C
Gas flow rate	He carrier gas, 13 mL min^{-1} Ar–methane, make-up gas, 40 mL min^{-1}
Injection volume	2 μL

Operating conditions for carfentrazone-ethyl confirmation

Instrument	HP 5890 or 6890 gas chromatograph
Column	DB-35MS, phenyl/methyl (35 : 65) silicone gum, 15 m × 0.25-mm i.d., 0.25-μm film thickness
Inlet	Splitless injection mode (cyclo-double gooseneck insert)
Detector	HP 5972 mass-selective detector
Temperatures	
Injection port	250 °C
Oven	150 °C/1 min (initial); 12.5 °C min^{-1} (ramp); 280 °C/10 min (final)

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Gas flow rate	He carrier gas, 1 mL min^{-1}
Injection volume	2 μL
Ions monitored	m/z 312, 340, and 411

Operating conditions for determination of acid metabolites

Instrument	HP 5890 gas chromatograph
Column	DB-35, phenyl/methyl (35 : 65) silicone gum, 15 m × 0.25-mm i.d., 0.25-μm film thickness
Inlet	Splitless injection mode (cyclo-double gooseneck insert)
Detector	HP 5972 mass-selective detector
Temperatures	
Injection port	250 °C
Oven	150 °C/1 min (initial); 15 °C min^{-1} (ramp); 280 °C/18 min (final)
Gas flow rate	He carrier gas, 1 mL min^{-1}
Injection volume	2 μL
Ions monitored	m/z 348 (DM-C-Cl-PAc derivative); m/z 362 (C-Cl-PAc derivative); m/z 413 (HM-C-CI-PAc derivative)

7 Method validation and quality control

7.1 Experimental design

The analytical method was validated at the LOQ (0.05 mg kg^{-1}) for each analyte by satisfactory recoveries of the respective analytes from control samples that were fortified at the initiation of each analysis set. The fortified control samples were carried through the procedure with each analysis set. An analysis set consisted of a minimum of one control sample, one laboratory-fortified control sample, and several treated samples.

A calibration curve was generated for each analyte at the initiation of the analytical phase of the study. Standard solutions for injection contained carfentrazone-ethyl or derivatized acid metabolites. Standard solutions were injected at the beginning of each set of assays and after every two or three samples to gage the instrument response.

7.2 Preparation of standards

Carfentrazone-ethyl, C-Cl-PAc, C-PAc, DM-C-Cl-PAc and HM-C-Cl-PAc stock solutions of 1000 μg mL^{-1} were prepared by dissolving the appropriate amounts of the analytical standards in acetonitrile. Working solutions were prepared in volumetric flasks by appropriate dilutions of the stock solutions for each analyte or combination of analytes. Working solutions containing the parent were prepared only in acetonitrile and working solutions containing acid metabolites were prepared in acetonitrile (underivatized) or hexane (derivatized). Underivatized solutions (containing the parent and/or metabolites in acetonitrile) were used for fortification. Solutions of derivatized esters were prepared simultaneously with the samples. Standard solutions

of carfentrazone-ethyl (in acetonitrile) and derivatized acid metabolites (in hexane) were used for analyte quantitation and instrument calibration.

7.3 Calculation

The amounts of carfentrazone-ethyl, C-Cl-PAc, C-PAc, DM-C-Cl-PAc and HM-C-Cl-PAc were quantitated by the external standard calibration method.

The amount of sample injected was determined using the following equation:

$$\text{Amount of sample injected (mg)} = \frac{\text{initial aliquot weight (mg)}}{\text{final sample extract volume (}\mu\text{L)}} \times \text{sample extract volume injected (}\mu\text{L)}$$

An equation representing area versus concentration was determined using a standard linear regression analysis applied to the injection standards, yielding a slope m and an intercept b. The following equation was then used to calculate the concentration of the sample injected from the area measured:

$$\text{Concentration of sample (ng } \mu\text{L}^{-1}) = \frac{\text{Area of sample} - b}{m}$$

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The amount of analyte (in nanograms) detected in a sample injection was calculated by multiplying the concentration calculated above by the injection volume. Then the concentration detected (in ppm) was determined by dividing this result by the amount of sample injected:

$$\text{Detected or uncorrected ppm (ng mg}^{-1}) = \frac{\text{conc. of sample (ng } \mu\text{L}^{-1}) \times \text{inj. volume (}\mu\text{L)}}{\text{amount of sample injected (mg)}}$$

No correction for molecular weights was necessary for the derivatized compounds since the injection standards were derivatized simultaneously with the analytes and all weights were based on the underivatized acids.

The uncorrected ppm of the fortified control samples was divided by the fortification level and multiplied by 100% to calculate the method recovery (%). The following equation was used:

$$\text{Method recovery (\%)} = \frac{\text{uncorrected mg kg}^{-1} - \text{control mg kg}^{-1}}{\text{fortification level (mg kg}^{-1})} \times 100$$

The LOD was calculated as the concentration of analyte (ppm equivalent) at one-fifth the area of the LOQ level standard, or one-fifth the LOQ, whichever was larger.

7.4 Time required for analysis

For a set of 10 samples, the analytical method can be completed within 16 laboratory hours from the time of sample weighing to GC injection.

7.5 Accuracy and precision

The accuracy and precision of the analytical methods were determined by the average and standard deviation of individual method recoveries of the fortified-control samples in 50 different matrices (see Tables 1 and 2). These methods were also demonstrated to be very rugged based on the results of accuracy and precision for a variety of crop and animal matrices.

8 Important points

The extraction efficiencies using a blender and a shaker were compared and both methods gave similar results. A corn sample treated with radiolabeled carfentrazone-ethyl and collected from a metabolism study was used for comparison. Multiple samples can be extracted simultaneously if extraction is performed by shaking. In addition, since the extraction procedures in the residue study closely followed the extraction scheme in the metabolism study, the resulting extraction efficiencies from both studies were almost identical.

During the initial partition with hexane and water, the aqueous pH must not exceed 8. Carfentrazone-ethyl is extremely unstable under alkaline conditions and will rapidly degrade to C-Cl-PAc. At times, the workup of the crop samples, including the fortification step, should be completely separated for carfentrazone-ethyl and the acid metabolites, to avoid any possible interference from the parent compound.

Both the washing solvent and the volume of it used during the SI cleanup step were critical to the method recovery. Generally, different volumes of wash solvents were needed in different methods to reduce the amount of co-extracts present without jeopardizing the recovery of the analytes. Silica gel cartridges from Varian were used to analyze the crop and animal matrices. When cartridges from other manufacturers were used, different elution patterns were observed. Therefore, the cartridge elution pattern should be evaluated prior to usage.

Pyridine and BF_3 in methanol are hazardous and must be used only in a well-ventilated hood. A solvent partition after acylation helps remove residual pyridine from the sample. Material Safety Data Sheets for the derivatizing agents should be reviewed and kept readily available.

The injection standards of carfentrazone-ethyl must be in acetonitrile. Other solvents (e.g., ethyl acetate) lead to poor chromatography following injection of matrix samples. This can lead to apparent enhanced recoveries of analyte in the fortified samples.

Conditioning the GC system with matrix samples before the actual run of the set is recommended to establish stable analytical conditions for the analytes. The GC

Table 1 Recoveries from fortified samples

Matrix	Fortification level (mg kg^{-1})	No. of analyses	% Recovery (average ± SD)			
			Carfentrazone-ethyl	C-Cl-PAc	DM-C-Cl-PAc	HM-C-Cl-PAc
Field corn grain	0.05	23	88 ± 9	93 ± 11	92 ± 10	NA[a]
Field corn forage	0.05, 0.1, 0.15, 0.2, 0.3	14, 22, 23	98 ± 15	89 ± 15	87 ± 14	87 ± 12
Field corn fodder	0.05, 0.1, 0.3	9, 21, 22	90 ± 15	93 ± 11	86 ± 17	101 ± 16
Field corn grits	0.05	2	72	105	103	NA
Field corn meal	0.05	2	76	110	105	NA
Field corn flour	0.05	2	95	100	85	NA
Field corn starch	0.05	2	93	85	83	NA
Field corn crude oil	0.05	2	97	80	109	NA
Field corn refined oil	0.05	5	92 ± 18	79 ± 7	75 ± 4	NA
Sweet corn ears	0.05	8	94 ± 9	103 ± 11	104 ± 9	NA
Sweet corn forage	0.05, 0.1	8	86 ± 6	100 ± 11	99 ± 15	NA
Sweet corn fodder	0.05, 0.2	8, 9	88 ± 8	96 ± 9	96 ± 16	NA
Wheat grain	0.05	8	89 ± 14	93 ± 10	93 ± 15	NA
Wheat forage	0.05, 0.25, 0.5	6	99 ± 4	98 ± 13	78 ± 12	101 ± 11
Wheat hay	0.05, 0.25	3	99 ± 8	89 ± 15	86 ± 14	95 ± 19
Wheat straw	0.05, 0.25	5	104 ± 10	89 ± 15	87 ± 10	107 ± 16
Wheat bran	0.05	1	97	100	82	NA
Wheat flour	0.05	1	97	79	67	NA
Wheat middlings	0.05	1	68	105	74	NA
Wheat shorts	0.05	1	108	93	85	NA
Wheat germ	0.05	1	114	81	76	NA
Sorghum grain	0.05	13	97 ± 16	95 ± 8	92 ± 14	NA
Sorghum forage	0.05, 0.1	6	108 ± 10	108 ± 13	100 ± 8	NA
Sorghum fodder	0.05	7	94 ± 10	101 ± 10	100 ± 8	NA
Sorghum flour	0.05	2	116	85	97	NA
Soybean seed	0.05	12	91 ± 10	96 ± 21	NA	92 ± 14
Soybean forage	0.05, 0.25, 1.0	4, 5	105 ± 9	90 ± 11	NA	101 ± 6
Soybean hulls	0.05	1	108	89	NA	120
Soybean meal	0.05	1	98	126	NA	117
Soybean crude oil	0.05	1, 2	117	92	NA	101
Soybean refined oil	0.05	2	117	81	NA	64
Rice grain	0.05	21, 22	91 ± 11	102 ± 11	106 ± 11	NA
Rice straw	0.05, 0.1, 1.0, 5.0	18, 21	98 ± 14	94 ± 12	89 ± 15	98 ± 14
Rice hulls	0.05	2	105	103	99	NA
Rice bran	0.05	2	103	79	78	NA
Rice, polished	0.05	2	110	104	104	NA
Cotton seed	0.05, 0.1, 10	12, 14	94 ± 16	76 ± 12	NA	88 ± 21
Cotton gin trash	0.05, 10	6, 7	89 ± 23	82 ± 17	NA	90 ± 17
Cotton meal	0.05, 0.1	3	99 ± 9	86 ± 11	NA	100 ± 11
Cotton hulls	0.05, 0.1	3	104 ± 7	109 ± 13	NA	82 ± 9
Cotton refined oil	0.05, 0.1	3	125 ± 6	93 ± 10	NA	75 ± 12
Grapes	0.05, 0.1	7	100 ± 10	97 ± 15	79 ± 13	74 ± 9
Raisins	0.1	1	99	98	82	67

[a] NA, not analyzed.

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Table 2 Recoveries from fortified samples

Matrix	Fortification level (mg kg^{-1})	No. of analyses	% Recovery (average ± SD)		
			Carfentrazone-ethyl	C-Cl-PAc	C-PAc
Bovine milk	0.025, 0.25	12, 20	88 ± 11	92 ± 18	90 ± 14
Bovine milk cream	0.05	2	77	73	68
Bovine liver	0.05	2	NA[a]	81	90
Bovine muscle	0.05	2	NA	89	100
Bovine kidney	0.05, 0.5	4, 6	91 ± 4	80 ± 8	87 ± 21
Bovine fat	0.05	2	102	108	104

[a] NA, not analyzed.

oven is programmed to a high final temperature after the analysis run to bake out any possible late eluting compounds.

More recently, liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) have been evaluated as possible alternative methods for carfentrazone-ethyl compounds in crop matrices. The LC/MS methods allow the chemical derivatization step for the acid metabolites to be avoided, reducing the analysis time. These new methods provide excellent sensitivity and method recovery for carfentrazone-ethyl. However, the final sample extracts, after being cleaned up extensively using three SPE cartridges, still exhibited ionization suppression due to the matrix background for the acid metabolites. Acceptable method recoveries (70–120%) of carfentrazone-ethyl metabolites have not yet been obtained.

9 Storage stability

Storage stability studies for carfentrazone-ethyl compounds on crop matrices have shown a pattern of stability for at least 7–24 months, depending on the study program or the maximum sample storage interval for the study. Carfentrazone-ethyl was not stable in field corn starch, potato tuber and bovine kidney. The residue results indicated that a significant portion of carfentrazone-ethyl was converted to C-Cl-PAc in these matrices; however, the total amount of carfentrazone-ethyl and C-Cl-PAc accounted for the original spiking level. Since both carfentrazone-ethyl and C-Cl-PAc were determined in these stability studies, the instability of carfentrazone-ethyl was not of any concern.

Acknowledgements

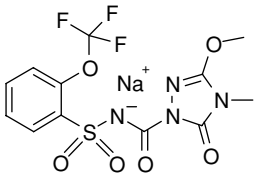
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Flucarbazone-sodium

Materials to be analyzed	Wheat forage, wheat hay, wheat straw, wheat grain, soil and water
Instrumentation	Liquid chromatography/tandem mass spectrometry (LC/MS/MS) for crop, soil and water analysis

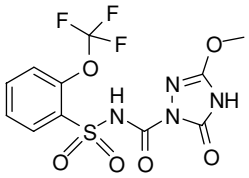
1 Introduction

Chemical name (IUPAC)	4,5-Dihydro-3-methoxy-4-methyl-5-oxo-*N*-[[2-(tri-fluoromethoxy)phenyl]sulfonyl]-1*H*-1,2,4-triazole-1-carboxamide, sodium salt
Structural formula	
Empirical formula	$C_{12}H_{10}F_3N_4NaO_6S$
CAS No.	145026-88-6
Molar mass	418.3
Melting point	200 °C (decomposes)
Boiling point	Not applicable
Vapor pressure	Nonvolatile
Solubility	Water 44 g L^{-1} at 20 °C Dichloromethane 0.72 g L^{-1} 2-Propanol 0.27 g L^{-1} Acetone 1.3 g L^{-1} Ethyl acetate 0.14 g L^{-1} Acetonitrile 6.4 g L^{-1} Dimethyl sulfoxide >250 g L^{-1}
Stability	Stable in neutral to basic aqueous solution Unstable in acidic aqueous solution
Use pattern	Flucarbazone-sodium is a post-emergent grass herbicide for use in winter wheat and spring wheat, including

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	durum. This ALS inhibitor herbicide has superior control of wild oats and green foxtail, including resistant biotypes
Regulatory position	The crop residue definition includes parent flucarbazone-sodium and the *N*-desmethyl flucarbazone metabolite (**I**). The soil and water residue definitions include parent flucarbazone-sodium and the sulfonic acid, sulfonamide and *N,O*-dimethyl triazolinone (NODT) metabolites



(**I**)

2 Outline of method

Wheat samples are extracted with dilute ammonia on the ASE200. The extracts are amended with isotopically labeled internal standards. The extracts are purified by sequential octadecyl reversed-phase solid-phase extraction (C_{18} SPE) and ethylenediamine-*N*-propyl anion exchange (PSA) SPE. The samples are analyzed by LC/MS/MS. This method determines crop residues of flucarbazone-sodium and *N*-desmethyl flucarbazone with a limit of quantitation (LOQ) of 0.01 mg kg^{-1} for each analyte.

Soil samples are extracted with buffered acetonitrile with a mechanical shaker. After centrifuging, aliquots of the extracts are amended with isotopically labeled internal standards and evaporated to dryness. The samples are reconstituted and analyzed by LC/MS/MS. This method determines soil residues of flucarbazone-sodium, sulfonic acid, sulfonamide and NODT with an LOQ of 0.001 mg kg^{-1} for each analyte.

Water samples are amended with isotopically labeled internal standards, acidified, and purified/concentrated by C_{18} SPE. The extracts are evaporated to dryness, reconstituted with mobile phase and analyzed by LC/MS/MS. This method determines water residues of flucarbazone-sodium, sulfonic acid, sulfonamide and NODT with an LOQ of 50 ng kg^{-1} for each analyte.

3 Apparatus

Assorted laboratory glassware
Dionex Accelerated Solvent Extractor (ASE200) with 11- and 22-mL extractor cells
Laboratory mechanical shaker
Laboratory centrifuge (IEC Centra-8 or equivalent)
N-EVAP or TurboVap LV sample evaporator

Reversed-phase C_8 chromatography column, Zorbax SB-C8, 150 × 4.6-mm i.d., 3.5-μm particle size
Reversed-phase C_{18} chromatography column, Keystone Scientific Betasil, 100 × 2.0-mm i.d., 5-μm particle size, 100 A, Part No. 105-701-2-CPF
TSQ 7000 LC/MS/MS system with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) interface and gradient high-performance liquid chromatography (HPLC) unit, or equivalent
Vacuum manifold for use with SPE cartridges (Varian Vac Elut 10 or equivalent)

4 Reagents and consumable supplies

Acetic acid, glacial (HOAc)
Acetonitrile (ACN), HPLC grade
Ammonium acetate (NH_4OAc), reagent grade
Ammonium hydroxide, 28%, reagent grade
Autosampler vials and septa caps
Celite, or equivalent
Disposable vials, 60-mL volume (I-Chem, Cat. No. S236-0060)
PSA SPE cartridges, 0.5-g/3-mL (Varian Bond Elut, Cat. No. 1410-2042).
Formic acid, 88%, reagent grade
Hydrochloric acid (HCl), concentrated, reagent grade
Methanol (MeOH), HPLC grade
Nylon syringe filter, 0.45-μm, 25-mm
C_{18} SPE cartridges, 1-g/6-mL, Varian Mega Bond Elut, Cat. No. 1225-6001, or equivalent
C_{18} SPE cartridges, 2-g/12-mL, Varian Mega Bond Elut, Cat. No. 1225-6015, or equivalent
Paper filter disks for the ASE (Dionex, Cat. No. 049458)
Water, HPLC grade, or equivalent

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Soil extraction solvent is prepared by dissolving 15.4 g of NH_4OAc in 1 L of 0.125 N aqueous HCl and mixing the resultant solution with 4 L of ACN.

5 Sampling and preparation

Wheat samples should be sampled and prepared for analysis according to the general instructions provided in the 'Pesticide Analytical Manual', Vol. 1.[1] Soil samples should be prepared for analysis by homogenization with a hammer mill or knife mill. Water samples are used without sample preparation.

6 Procedure

6.1 Extraction

6.1.1 Wheat samples

For wheat forage, hay and straw, 1.00 g of homogenized sample is weighed directly into an 11-mL extractor cell containing a paper filter disk. The sample is extracted

with 0.05 M aqueous NH_4OH with the ASE200 under the following conditions: extraction temperature 70 °C; pressure 1500 psi; three 5-min static cycles; 100% flush volume; 150-s purge time. The extract is collected in a 60-mL vial and amended with 0.100 mL of a solution containing 1.00 μg mL^{-1} of both flucarbazone-d_3 and *N*-desmethyl flucarbazone-d_3.

For wheat grain, 1.00 g of homogenized sample is weighed into a 100-mL beaker and mixed with 4 g of Celite. The mixture is added to a 22-mL extractor cell containing a paper filter disk and 0.5 g of Celite. The sample is extracted with 0.05 M aqueous NH_4OH with the ASE200 under the following conditions: extraction temperature 70 °C; pressure 1500 psi; three 5-min static cycles; 60% flush volume; 150-s purge time. The extract is collected in a 60-mL vial and amended with 0.2 g of Celite and 0.100 mL of an ACN solution containing 1.00 μg mL^{-1} of both flucarbazone-d_3 and *N*-desmethyl flucarbazone-d_3.

6.1.2 Soil

A 10.0-g soil sample is weighed into a 60-mL vial and 20 mL of soil extraction solvent are added. The vial is capped and shaken horizontally in an orbital shaker for 1 h. The sample is centrifuged at 1500 rpm for 5 min. A 4.0-mL aliquot of the supernatant is transferred to a test-tube containing 0.100 mL of an ACN solution containing 100 ng mL^{-1} each of flucarbazone-d_3, sulfonic acid-d_3, sulfonamide-d_3 and NODT-d_3. The solvent is removed on an N-EVAP or TurboVap (40–45 °C). The residue is dissolved in 1.0 mL of water–0.1 M NH_4OAc in MeOH (19 : 1, v/v). An aliquot of the sample is passed through a 0.45-μm nylon syringe filter into an autosampler vial.

6.1.3 Water

There is no specific extraction for water samples.

6.2 Cleanup/concentration

6.2.1 Wheat samples

The wheat extract is acidified with 0.50 mL of HOAc and loaded on to a C_{18} SPE cartridge (1-g/6-mL). The cartridge is washed with 5 mL of 0.1% HOAc. The cartridge is then eluted with 5 mL of ACN–0.1% HOAc (3 : 1, v/v) into a test-tube. The eluate is diluted to 10 mL with 0.1% HOAc. The resultant solution is loaded on to a PSA SPE cartridge. The PSA cartridge is washed with 5 mL of 0.1% HOAc. The PSA cartridge is eluted with 5 mL of 0.5 M NH_4OH into a test-tube. Just before analysis, the sample is acidified with 0.15 mL of HOAc.

6.2.2 Soil

No specific cleanup is needed for soil samples.

6.2.3 Water

A 50-mL water sample is acidified with 1 mL of 1 N HCl and amended with 0.050 mL of an ACN solution containing 100 ng mL^{-1} each of flucarbazone-d_3, sulfonic acid-d_3, sulfonamide-d_3 and NODT-d_3. The sample is loaded on to a C_{18} SPE cartridge (2-g/12-mL). The cartridge is washed with 10 mL of water. The cartridge is eluted with 10 mL of MeOH–5% NH_4OH (9 : 1, v/v). The solvent is removed on an N-EVAP or TurboVap (40–45 °C). The residue is dissolved in 1.0 mL of water–0.1 M NH_4OAc in MeOH (19 : 1, v/v). An aliquot of the sample is passed through a 0.45-μm nylon syringe filter into an autosampler vial.

6.3 Chromatographic determination

6.3.1 Plant material

HPLC conditions

Instrument	Thermo Separation Consta Metric 3200 MS
Column	Zorbax SB-C8, 150 × 4.6-mm i.d., 3.5-μm particle size
Flow rate	0.8 mL min^{-1} with a 3 : 1 split
Injection volume	200 μL
Mobile phase A	0.1% aqueous formic acid
Mobile phase B	MeOH
Gradient	68% A at 0 min, linear gradient to 35% A at 10 min, linear gradient to 5% A at 10.5 min, hold at 5% A until 13.5 min, then linear gradient back to 68% A

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Mass spectrometry (MS) conditions

Instrument	Finnigan TSQ 7000
Data collection	Data are collected for negative daughter ion transitions of m/z 381 to 114 and m/z 381 to 99 for the *N*-desmethyl flucarbazone analyte and m/z 384 to 117 for the *N*-desmethyl flucarbazone-d_3 internal standard Data are collected for negative daughter ion transitions of m/z 395 to 128 and m/z 381 to 113 for the flucarbazone-sodium analyte and m/z 395 to 131 for the flucarbazone-sodium-d_3 internal standard

Note: The LC/MS/MS system should be set up and tuned by a skilled operator for maximum sensitivity to flucarbazone-sodium and *N*-desmethyl flucarbazone

6.3.2 Soil and water

HPLC conditions

Instrument	Thermo Separation Consta Metric 3200 MS
Column	Betasil C_{18}, 100 × 2-mm i.d., 5-μm particle size, 100 A (Keystone Scientific)
Flow rate	0.3 mL min^{-1} with a 3 : 1 split

Injection volume	50 μL
Mobile phase A	Water–0.1 M NH_4OAc in MeOH (19 : 1, v/v)
Mobile phase B	5 mM NH_4OAc in MeOH
Gradient	100% A at 0 min, linear gradient to 5% A at 6 min, hold at 5% A until 7 min, then linear gradient back to 100% A to re-equilibrate the HPLC column

MS conditions

Instrument	Finnigan TSQ 7000
Data collection	Data are collected for positive daughter ion transitions of m/z 130 to 115 for the NODT analyte and m/z 133 to 115 for the NODT-d_3 internal standard Data are collected for negative daughter ion transitions of m/z 241 to 85 for the sulfonic acid analyte and m/z 244 to 85 for the sulfonic acid-d_3 internal standard Data are collected for negative daughter ion transitions of m/z 395 to 113 for the flucarbazone-sodium analyte and m/z 398 to 113 for the flucarbazone-sodium-d_3 internal standard Data are collected for negative daughter ion transitions of m/z 240 to 85 for the sulfonamide analyte and m/z 243 to 85 for the sulfonamide-d_3 internal standard

Note: The LC/MS/MS system should be set up and tuned by a skilled operator for maximum sensitivity to flucarbazone-sodium and the soil and water metabolites

7 Evaluation

7.1 Method

7.1.1 Wheat

The wheat sample residue level is determined from the relative mass spectral responses of the analytes to the corresponding isotopically labeled internal standards. The sample relative response is compared with the average relative response of a standard solution of analyte and internal standard analyzed before and after the sample (bracketing standards). Both samples and standards receive the same amount, 100 ng, of each internal standard to facilitate the comparison. The calculations to determine the residue level in wheat tissues are outlined in Section 7.3.1.

7.1.2 Soil

The soil residue level is determined from the relative responses of the analytes to the internal standards. A five-point calibration curve is analyzed in triplicate, and the data are analyzed by a weighted $1/x$ linear regression model. The calculated slope and intercept from the linear regression are used to calculate the residue levels in the soil samples. A 20% aliquot of the sample extract receives 10 ng of each internal standard

(equivalent to 5 ng g^{-1} of original sample). The calculations to determine the residue level in soil are outlined in Section 7.3.2.

7.1.3 Water

The water residue level is also determined from the relative responses of the analytes to the internal standards. The sample residue levels are calculated by comparison with an average response factor determined by triplicate analysis of a five-point calibration curve. Samples receive 5 ng of each internal standard (0.1 ng mL^{-1}) and are concentrated 50-fold by C_{18} SPE before analysis to achieve adequate instrumental sensitivity. The calculations to determine the residue level in water are outlined in Section 7.3.3.

7.2 Recoveries, limit of detection and limit of quantitation

7.2.1 Wheat

The average recoveries for flucarbazone-sodium at the 0.01 mg kg^{-1} fortification level were 91% for forage, 92% for hay, 93% for straw and 95% for grain. The average recoveries for *N*-desmethyl flucarbazone at the 0.01 mg kg^{-1} fortification level were 88% for forage, 85% for hay, 87% for straw and 93% for grain. The average recoveries for flucarbazone-sodium at the 0.10 mg kg^{-1} fortification level were 96% for forage, 91% for hay and 88% for straw. The average recoveries for *N*-desmethyl flucarbazone at the 0.10 mg kg^{-1} fortification level were 94% for forage, 89% for hay and 94% for straw.

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Because acceptable recoveries were obtained at 0.01 mg kg^{-1}, the method LOQ is 0.01 mg kg^{-1} for flucarbazone-sodium and 0.01 mg kg^{-1} for *N*-desmethyl flucarbazone in all wheat samples. Linearity curves in both solvent and matrix blanks were run from 0.005 to 0.100 mg kg^{-1}. Because 0.005 mg kg^{-1} of each analyte was reliably detected, the method limit of detection (LOD) was 0.005 mg kg^{-1} in all wheat samples.

7.2.2 Soil

Five replicate recoveries of flucarbazone-sodium, sulfonic acid, sulfonamide and NODT from soil fortified at 0.001 mg kg^{-1} averaged 97, 90, 100 and 87%, respectively. Therefore, the LOQ is 0.001 mg kg^{-1} for each analyte.

7.2.3 Water

Seven replicate recoveries of flucarbazone-sodium, sulfonic acid, sulfonamide and NODT from well water fortified at 50 ng L^{-1} averaged 106, 100, 89 and 106%, respectively. Therefore, the LOQ is 50 ng L^{-1} for each analyte. The method detection limits for flucarbazone-sodium, sulfonic acid, sulfonamide and NODT, as determined by the United States Environmental Protection Agency (USEPA) recommended technique,[2] are 5, 11, 20 and 19 ng L^{-1}, respectively.

7.3 Calculation of residues

7.3.1 Wheat

Determine the relative responses for the analytes in the sample and in the bracketing standards by the following equation:

$$\text{Relative response} = \frac{\text{analyte peak area}}{\text{internal standard peak area}}$$

Average the relative response values for the two standard solution analyses. Use the following equation to determine the residue levels in the sample:

$$\text{Residue level (ppm)} = \frac{\text{sample relative response}}{\text{average standard relative response}} \times \frac{\text{standard concentration}}{\text{exact sample weight}}$$

where

analyte peak area = mass spectral response to flucarbazone-sodium or to *N*-desmethyl flucarbazone

internal standard peak area = mass spectral response to flucarbazone-sodium-d_3 or to *N*-desmethyl flucarbazone-d_3

standard concentration = amount (μg) of flucarbazone-sodium or *N*-desmethyl flucarbazone in solution per 100 ng of the corresponding internal standard (the amount of internal standard added to the extract of a 1-g sample)

7.3.2 Soil

Determine the relative responses for the calibration curve analyses by the following equation:

$$\text{Relative response} = \frac{\text{analyte peak area}}{\text{internal standard peak area}}$$

Perform a $1/x$ weighted linear regression analysis of relative response versus standard concentration in ng per 5 ng of internal standard. Calculate the slope and intercept values from the regression analysis. Use the following equation to determine the residue levels in the sample:

$$\text{Residue level (ppb)} = \frac{(\text{sample relative response} - \text{intercept})(\text{20-mL extract volume})}{(\text{slope})(\text{sample weight})(\text{4.0-mL aliquot volume})}$$

where

analyte peak area = mass spectral response to the analyte

internal standard peak area = mass spectral response to corresponding internal standard

sample weight = actual sample weight (nominally 10 g)

7.3.3 Water

Determine the response factors for the calibration curve analyses by the following equation:

$$\text{Relative response} = \frac{\text{analyte peak area}}{(\text{internal standard peak area})(\text{standard concentration})}$$

Calculate an average value from the response factors obtained from the calibration curve analyses. Use the average response factor in the following equation to determine the residue levels in the sample:

$$\text{Residue level (ppb)} = \frac{\text{analyte peak area}}{(\text{internal standard peak area})(\text{average response factor})}$$

where

analyte peak area = mass spectral response to the analyte
internal standard peak area = mass spectral response to corresponding internal standard
standard concentration = amount (ng) of analyte in solution per 0.1 ng of the corresponding internal standard (the amount of internal standard added per milliliter of the original water sample)

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8 Important points

LC/MS/MS is used to measure the ratio of analyte to internal standard in the sample and standard (the relative response determination). Once the internal standard has been added to the sample extract or standard solution, the analyte/IS ratio will not change. Subsequent sample losses will not change the analyte/IS ratio, nor will sample dilutions, solvent evaporation, changes in instrumental response or loss of chromatographic resolution.

References

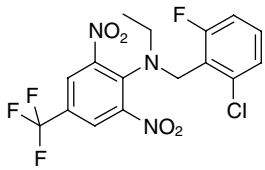
1. 'Pesticide Analytical Manual. Volume 1: Multiresidue Methods,' US Department of Health and Human Services, Washington, DC (1994).
2. 'Environmental Protection Agency Instruction 10 CFR Ch. 1 (7-1-94 Edition) Appendix B to Part 136 – Definition and Procedure for the Determination of the Method Detection Limit – Revision 1.11,' US Environmental Protection Agency, Washington, DC (1994).

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Flumetralin

Materials to be analyzed	Soil and tobacco
Instrumentation	Gas chromatography with electron capture and/or mass-selective detection

1 Introduction

Chemical name (IUPAC)	*N*-(2-Chloro-6-fluorobenzyl)-*N*-ethyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine
Structural formula	
Empirical formula	$C_{16}H_{12}ClF_4N_3O_4$
Molar mass	421.7
Melting point	92.4–103.7 °C
Vapor pressure	3.20×10^{-5} mbar at 25 °C
Solubility (25°C)	Water 70 μg L^{-1}, acetone 560 g L^{-1}, ethanol 18 g L^{-1}, toluene 400 g L^{-1}, n-hexane 14 g L^{-1}, n-octanol 6.8 g L^{-1}
Other properties	Yellow to orange odorless crystalline solid; density 1.54 g cm^{-3} at 20 °C; octanol/water partition coefficient (log *P*) 5.45 at 25 °C
Use pattern	Flumetralin is a plant growth regulator, used to control sucker growth in tobacco
Regulatory position	The definition of residue of regulatory concern is the parent, flumetralin, only

2 Outline of methods[1]

A soil sample (10 g) was extracted by mechanically shaking with methanol–deionized water. This mixture was filtered and a portion was removed for partitioning into toluene–hexane. A phenyl solid-phase extraction (SPE) cartridge was employed

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for additional cleanup only when interferences were encountered. Plant material (tobacco) was mixed with Florisil and extracted using hexane in a Soxhlet extractor. A portion of the soil and plant extract was directly analyzed using gas chromatography/electron capture detection (GC/ECD) or gas chromatography/mass spectrometry (GC/MS).

3 Apparatus

Mechanical shaker
Mixing device (e.g. vortex)
N-Evaporator
Phenyl SPE cartridge (Analytichem, Bond Elut)
Soxhlet extractor
Visiprep SPE manifold
Visiprep drying attachment

4 Reagents

Ammonia solution, 25% aqueous
Florisil (5% deactivated)
Hexane
Methanol
Methyl *tert*-butyl ether (MTBE)
Toluene
Water, high-performance liquid chromatography (HPLC) grade

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5 Sample preparation

5.1 Soil

Flumetralin was extracted from soil by mechanically shaking 10 g of a representative soil sample with 100 mL of methanol–deionized water (4 : 1, v/v) for 2 h. After shaking, the soil particles were allowed to settle until the supernatant was clear, followed by filtering through filter-paper. A 15-mL aliquot portion of the clear supernatant was removed using a pipette and transferred to a 50-mL cylinder (with stopper). A 15-mL portion of saturated aqueous NaCl solution was added, followed by mixing. This fraction was partitioned with 3 mL of toluene–hexane (1 : 1, v/v) by shaking vigorously for 1–2 min on a vortex mixer followed by mechanical shaking for 10 min. A portion of the organic phase was removed for analysis using GC/ECD after phase separation. An SPE step was employed when interference was encountered using the partitioning procedure. A phenyl SPE cartridge was conditioned with 5 mL of methanol followed by 5 mL of water. The cartridge was not allowed to go dry. The cartridge was loaded with 15 mL of the clear supernatant and allowed to percolate dropwise. The column was dried with a flow of nitrogen for 30 min followed by

elution of the analyte with three times 3 mL of hexane–MTBE (4 : 1, v/v). The eluate was reduced to about 2 mL and diluted to a final volume of 3 mL using hexane prior to final analysis.

5.2 Plant materials

Flumetralin was extracted from tobacco using Soxhlet extraction. A 5-g amount of Florisil (5% deactivated) was transferred directly on to the filter disk of a Soxhlet extractor followed by another 5 g of Florisil mixed with 5 g of ground tobacco sample as an upper layer. A 60-mL volume of hexane and 3 mL of a 4 $\mu g\,mL^{-1}$ internal standard solution were placed in a 250-mL round-bottom flask prior to attaching the Soxhlet extractor. The unit was placed on a heating mantle and the hexane was refluxed through the extractor at the rate of about 250 $mL\,h^{-1}$ for 4.5 h. After cooling, 0.5 μL of the extract was injected directly into a GC/ECD or GC/MS system.

6 Instrumentation

The following instrumental conditions have been shown to be suitable for the analysis of flumetralin. Other operating parameters may be employed provided that flumetralin is separated from sample interference and the response is linear over the range of interest.

Operating conditions for soil

Gas chromatograph	Hewlett-Packard, Model 5890 with electron-capture detector
Column	DB-5 fused silica, 15 m × 0.53-mm i.d. (a DB-1 or DB-17 column may also be used)
Column temperature	190 °C (12 min) to 240 °C (4 min)
Injection port temperature	250 °C
Detector	Electron capture detector, temperature 300 °C
Gas flow rates	Carrier gas, hydrogen at 10 $mL\,min^{-1}$ Detector gas, nitrogen at 64 $mL\,min^{-1}$
Injection volume	2 μL for capillary column
Retention time for flumetralin	8.6 min (11.9 min on DB-17)

If GC/MS is used, the diagnostic ions are m/z 218 (target) and 361 (molecular ion)

Operating conditions for tobacco

Gas chromatograph	Hewlett-Packard, Model 5890 with ^{63}Ni electron capture detector
Column	DB-5 fused silica, 30 m × 0.32-mm i.d.
Column temperature	70 °C (held 1 min), increases at 20 °C min^{-1} to 150 °C then at 3 °C min^{-1} to 270 °C (held 15 min)
Injection port temperature	Not specified

Detector	Not specified
Gas flow rates	Carrier gas, helium at 4 mL min^{-1}
	Detector gas, nitrogen at 30 mL min^{-1}
Injection volume	0.5 µL
Retention time for flumetralin	23.5 min (34.2 min)

7 Evaluation

7.1 Method

Quantitation was performed in all cases using the external calibration method. A series of standards were injected and the responses plotted against their known concentrations. Peak responses in samples were compared with the calibration plots to obtain the amount found (nanograms). A fresh calibration plot was generated with each analytical set of samples.

7.2 Recoveries, limit of detection (LOD) and limit of quantitation (LOQ)

The recoveries for flumetralin in soil ranged from 77 to 117% at the established method LOQ of 0.01 µg g^{-1}.

The average recovery for flumetralin in tobacco was 99% at a fortification level of 1.3 µg g^{-1}. The LOD was 0.1 µg g^{-1}.

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7.3 Calculation of residues

$$C = LWV_A / V_E V_F$$

where L = lower practical limit, W = weight of sample, V_A = volume of aliquot, V_E = total extract volume, and V_F = final fraction volume

µg g^{-1}(ppm) = ng found (from calibration plot)/mg sample injected

Reference

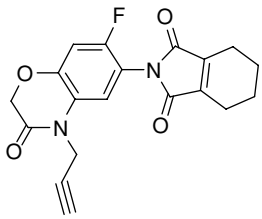
1. D. Amati and Y. Li, *J. Chromatogr.,* **539**, 237 (1991).

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Flumioxazin

Materials to be analyzed	Almond (nutmeat and hulls), ginned cottonseed, grape, peanut (vines, hay, nutmeat and hulls), soybean (forage, hay and seed), sugarcane, water, and soil
Instrumentation	Gas-chromatographic determination

1 Introduction

Chemical name (IUPAC)	*N*-(7-Fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide
Structural formula	
Empirical formula	$C_{19}H_{15}FN_2O_4$
Molar mass	354.3
Melting point	201.8–203.8 °C
Vapor pressure	2.41×10^{-6} mm Hg at 22.0 °C
Solubility	1.8 mg L^{-1} in water at 25 °C Soluble in polar organic solvents
Stability	Unstable in acidic, neutral and basic aqueous solutions
Use pattern	Flumioxazin provides pre-emergence control of susceptible weeds in crops. Flumioxazin may also be used as part of an early preplant burn-down program in various crops
Regulatory position	The residue of concern is the parent molecule, flumioxazin

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2 Outline of method

2.1 Plant matrices

Residues of flumioxazin are extracted from plant matrices with aqueous acetone. The extracted residues are partitioned into dichloromethane. The dichloromethane is removed through rotary evaporation. Partitioning between hexane–acetonitrile followed by Florisil column chromatography purifies the plant extract. Residues of flumioxazin are quantitated by gas chromatography GC.

2.2 Soil

Residues of flumioxazin in/on soil are extracted with acetone–0.1 N HCl (5 : 1, v/v), partitioned into dichloromethane, cleaned up with Florisil column chromatography and quantitated by GC.

2.3 Water

Flumioxazin is extracted from water with dichloromethane. If needed, the sample is cleaned with Florisil column chromatography prior to quantitation by GC.

3 Apparatus

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Column, chromatographic, 300 mm × 19-mm i.d. with Teflon stopcock
Filter flask, 500-mL
Round-bottom flask, 50-, 250- and 500-mL
Food chopper
Buchner funnel, 10-cm
Funnel, filter, 10-cm
Separatory funnel, 250-, 500- and 1000-mL
Gas chromatograph equipped with a nitrogen–phosphorus detector
Glass wool
Jar, 500-mL, with cap
Laboratory shaker
Wiley mill
Filter paper, Whatman No. 1, 9-cm.
Pasteur pipets
Rotary vacuum evaporator with 40 °C water-bath
Ultrasonic water-bath

4 Reagents

Acetone, pesticide grade
Acetonitrile, pesticide grade

Dichloromethane, pesticide grade
Ethyl acetate, pesticide grade
Florisil, PR grade, mixture, 2 parts 100–200 mesh plus 3 parts 60–100 mesh, dried overnight at 130 °C
Hexane, pesticide grade
Hydrochloric acid, reagent grade
Sodium chloride, reagent grade
Sodium sulfate, anhydrous, reagent grade
Water, deionized

5 Sampling and preparation

All crop samples should be prepared with a food chopper or Wiley mill to achieve a finely divided material. Soil and water samples should be well mixed to ensure a homogeneous sample.

6 Procedure

6.1 Extraction

6.1.1 Plant matrices

Combine 10 g of sample with 50 mL of acetone–water (4 : 1, v/v) in a 500-mL jar. Cap the jar and shake the sample on the laboratory shaker for 10 min. Allow the mixture to soak overnight at room temperature. Shake the sample for an additional 10 min and then filter the mixture into a 500-mL filter flask through a 10-cm Buchner funnel and Whatman No. 1 filter paper.

Return the filter cake to the extraction jar, add 50 mL of acetone–water (4 : 1, v/v) and shake the mixture on the laboratory shaker for 10 min. Filter the sample through a Buchner funnel and Whatman No. 1 filter paper, and combine the filtrate with the first extract. Rinse the filter cake with two 20-mL portions of acetone–water (4 : 1, v/v). Proceed to Section 6.2.1.

6.1.2 Soil

Weigh 20 g (wet weight) of soil into a 500-mL jar and add 50 mL of acetone–0.1 N HCl (5 : 1, v/v). Shake the sample for 10 min with a laboratory shaker, and then allow the sample to stand overnight at room temperature. Shake the sample for an additional 10 min and then filter the supernatant through a Buchner funnel and Whatman No. 1 filter paper.

Return the filter cake to the extraction jar and add 50 mL of acetone–0.1 N HCl (5 : 1, v/v). Shake the sample for 10 min, filter the supernatant through a Buchner funnel and Whatman No. 1 filter paper and combine this filtrate with the first extract. Rinse the filter cake with two 20-mL portions of acetone–0.1 N HCl solution (5 : 1, v/v). Proceed to Section 6.2.1.

6.1.3 Water

Measure 500 mL of water into a separatory funnel and add 150 mL of dichloromethane. Place the sample on a mechanical shaker and shake the funnel for 5 min. Drain the dichloromethane through a filter funnel containing ca 50 g of anhydrous sodium sulfate supported on a plug of glass wool. Re-extract the water sample with an additional 150 mL of dichloromethane for 5 min and filter the dichloromethane through anhydrous sodium sulfate. Combine the dichloromethane fractions and concentrate the extract to dryness in a rotary evaporator with a water-bath maintained below 40 °C. Proceed to Section 6.2.3.

6.2 Cleanup

6.2.1 Dichloromethane partition

Transfer the filtrate from Section 6.1.1 or 6.1.2 to a 500-mL separatory funnel and add 150 mL of 5% aqueous sodium chloride solution. Rinse the filter flask from the extraction procedure with two 40-mL portions of dichloromethane. Add both 40-mL rinses to the separatory funnel. Partition the residue into the dichloromethane. Filter the dichloromethane extract through a 10-cm filter funnel containing ca 50 g of anhydrous sodium sulfate supported on a plug of glass wool. Collect the dichloromethane in a 500-mL round-bottom flask. Repeat the partition and filtration steps with an additional 60 mL of dichloromethane. Rinse the sodium sulfate filter cake with 20 mL of dichloromethane and combine the partition and rinse solvents. Concentrate the combined dichloromethane solvents to dryness in a rotary evaporator under reduced pressure at ≤40 °C.

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For plant samples, proceed to Section 6.2.2.

For soil, after concentrating the dichloromethane, dissolve the dry residue in 1 mL of ethyl acetate and dilute the solution with 2 mL of hexane. Sonicate the contents of the flask for approximately 15 s to remove any residue remaining on the walls of the round-bottom flask. Proceed to Section 6.2.3.

6.2.2 Hexane–acetonitrile partition

Reconstitute the dry plant residue from Section 6.2.1 in 50 mL of hexane saturated with acetonitrile and transfer the flask contents to a 250-mL separatory funnel. Rinse the round-bottom flask with 50 mL of acetonitrile saturated with hexane and add this rinse to the hexane in the separatory funnel. Partition the residue from the hexane into the acetonitrile. Drain the acetonitrile into the 500-mL flask from the dichloromethane partition step (Section 6.2.1). Re-extract the remaining hexane phase with an additional 50 mL of acetonitrile saturated with hexane. Combine the acetonitrile fraction with the acetonitrile from the first partition. Concentrate the combined acetonitrile fractions to dryness in a rotary evaporator at ≤40 °C. Dissolve the dry residue in 1 mL of ethyl acetate and dilute the sample with 2 mL of hexane. Sonicate the sample for

approximately 15 s to remove any residue remaining on the walls of the round-bottom flask. Proceed to Section 6.2.3.

6.2.3 Florisil column cleanup

Note: each lot of Florisil must be checked for acceptable recovery of flumioxazin prior to initiating the column cleanup procedure. Adjust elution volumes and/or the solvent mixture as necessary to achieve >90% recovery for this step. Sample cleanup with Florisil may not be required for some water samples.

For all samples, prepare a chromatographic column with 15 g of Florisil and 40 mL of hexane–ethyl acetate (2 : 1, v/v). Drain the solvent to the top of the sorbent bed. Transfer the extract from the hexane–acetonitrile partition (Section 6.2.2) to the column with three 3-mL portions of hexane–ethyl acetate (2 : 1, v/v).

Rinse the column with 28 mL of hexane–ethyl acetate (2 : 1, v/v) and discard the rinse. Elute the flumioxazin with 70 mL of hexane–ethyl acetate (2 : 1, v/v), collecting the eluate in a 250-mL round-bottom flask. Evaporate the eluate to dryness and transfer the residue to a 50-mL round-bottom flask with three additional acetone rinses of 5 mL each. Concentrate the sample to dryness in a rotary evaporator under reduced pressure at ≤40 °C. Dissolve the residue in acetone for quantitation by GC.

6.3 Determination

Quantitate by GC.

Recommended operating conditions

Gas chromatograph	Hewlett-Packard (Agilent) 5890A
Injection port	Packed column port with a megabore column adapter
Temperature	275 °C
Carrier gas	Helium
Flow rate	10 mL min^{-1}
Column	Capillary, DB-17, 15 m × 0.53-mm i.d. 1.0-μm film thickness
Oven program	
Initial temperature	250 °C
Initial hold time	1 min
Ramp rate	20 °C min^{-1}
Final temperature	280 °C
Final hold time	8 min
Detector	Nitrogen–phosphorus detector
Temperature	300 °C
Auxiliary gas	Helium, 25 mL min^{-1}
Hydrogen	3.5 mL min^{-1}
Air	110 mL min^{-1}
Injection volume	1 μL
Retention time	5.7 min (approx.)

7 Evaluation

7.1 Method

Prior to use, the linearity of the GC system should be verified by analyzing at least four standards of different concentrations. The linearity standards should range in concentration from 0.1 to 2.0 $\mu g\,mL^{-1}$ for crops and soils and from 0.05 to 1.0 $\mu g\,mL^{-1}$ for water. The response of each standard is normalized to response per 1.0 $\mu g\,mL^{-1}$ by dividing the response of each standard by its concentration. The relative standard deviation (RSD) of these normalized responses should be $<10\%$.

Quantitation is performed using the external standard calibration technique. The concentration of the calibration standard in solution is 1.0 $\mu g\,mL^{-1}$. The calibration standard should be injected prior to injection of the treated samples and again after every second or third injection of treated samples. The analytical sequence should end with a calibration standard. The RSD of the calibration standards should be $<10\%$.

7.2 Recoveries, limit of detection and limit of quantitation

For plant samples, the average recovery of flumioxazin from untreated control samples fortified within the range 0.1–0.01 $mg\,kg^{-1}$ ranged from 75 to 106%. The limit of quantitation (LOQ) is 0.01 $mg\,kg^{-1}$ and the limit of detection (LOD) is 0.005 $mg\,kg^{-1}$.

For soil samples, the average recovery of flumioxazin from untreated control samples fortified within the range 0.05–0.01 $mg\,kg^{-1}$ was 100%. The LOQ is 0.01 $mg\,kg^{-1}$, and the LOD is 0.005 $mg\,kg^{-1}$.

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For water samples, the average recovery of flumioxazin from untreated control samples fortified within the range 0.05–5 $\mu g\,L^{-1}$ was 99%. The LOQ is 0.05 $\mu g\,L^{-1}$ and the LOD is 0.05 $\mu g\,L^{-1}$.

7.3 Calculation of residues

$$\text{Flumioxazin } (mg\,kg^{-1} \text{ or } \mu g\,L^{-1}) = \frac{A \times C \times V}{B \times W}$$

where

A = instrument response (integration counts) for flumioxazin in the sample
C = concentration of flumioxazin in the calibrating standard (1.0 $\mu g\,mL^{-1}$)
V = final volume of the sample extract (mL)
B = mean integration counts for the calibration standards
W = sample weight or volume (g or mL).

8 Important points

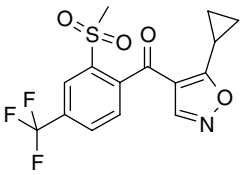
Each batch or lot of Florisil must be checked for recovery of flumioxazin prior to use in this method. If the recovery of flumioxazin through the Florisil is <90%, the elution volume and/or the solvent mixture utilized in Section 6.2.3 must be adjusted until suitable recoveries are obtained. The recovery through Florisil should be rechecked whenever method recovery values drop below acceptable values.

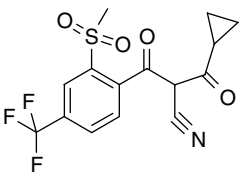
Thomas Schreier
Valent USA Corporation, Dublin, CA, USA

Isoxaflutole

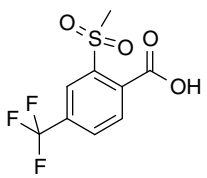
Materials to be analyzed	Groundwater and surface water
Instrumentation	Liquid chromatography/tandem mass spectrometry (LC/MS/MS).

1 Introduction

Chemical name (IUPAC)	5-Cyclopropyl-4-(2-methylsulfonyl-4-trifluoromethylbenzoyl)isoxazole
Structural formula	
Empirical formula	$C_{15}H_{12}F_3NO_4S$
Molecular weight	359.3
Melting point	135–136 °C
Vapor pressure	1×10^{-3} mPa (25 °C)
Water solubility	6.2 mg L^{-1} (pH 5.5, 20 °C)
Stability	Hydrolysis $t_{1/2}$, 1 day at pH 7
Use pattern	A broad-spectrum grass and broadleaf corn herbicide
Regulatory position	Regulated metabolites in groundwater include the parent, isoxaflutole (RPA 201772), and its metabolites RPA 202248 [2-cyclopropylcarbonyl-3-(2-methylsulfonyl-4-trifluoromethylphenyl)-3-oxopropanenitrile] and RPA 203328 (2-methanesulfonyl-4-trifluoromethylbenzoic acid)



RPA 202248



RPA 203328

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2 Outline of method

Residues of isoxaflutole, RPA 202248 and RPA 203328 are extracted from surface water or groundwater on to an RP-102 resin solid-phase extraction (SPE) cartridge, then eluted with an acetonitrile–methanol solvent mixture. Residues are determined by liquid chromatography/tandem mass spectrometry (LC/MS/MS) on a C_8 column. Quantitation of results is based on a comparison of the ratio of analyte response to isotopically labeled internal standard response versus analyte response to internal standard response for calibration standards.

3 Reagents and standards

Acetic acid, GR (EM Science, Cat. No. AX0073-13)
Acetonitrile, Omni-Solv (EM Science, Cat. No. AX0142-1)
Formic acid, Suprapur (EM Science, Cat. No. 11670-1)
Methanol, Omni-Solv (EM Science, Cat. No. MX0488-1)
Water, high-performance liquid chromatography (HPLC) grade

Analytes and ^{13}C-labeled internal standards

Isoxaflutole $^{13}C_6$-labeled internal standard:

Chemical name	Methanone, (5-cyclopropyl-4-isoxazolyl) [2-(methylsulfonyl)-4-(trifluoromethyl)phenyl-$^{13}C_6$]
Molecular weight	365.35

RPA 202248 $^{13}C_6$-labeled internal standard:

Chemical name	Benzene-$^{13}C_6$- propanenitrile, α-(cyclopropylcarbonyl)-2-(methylsulfonyl)-β-oxo-4-(trifluoromethyl)
Molecular weight	365.35

RPA 203328 $^{13}C_6$-labeled internal standard:

Chemical name	Benzoic acid-$^{13}C_6$, 2-(methylsulfonyl)-4-(trifluoromethyl)
Molecular weight	274.21

The molecular weights for the unlabeled isoxaflutole, RPA 202248 and RPA 203328 are 359.35, 359.35, and 268.21, respectively.

Note: isoxaflutole will degrade to RPA 202248 in solution. Standard solutions are stable for approximately 3 months when kept under refrigeration. A solution containing only isoxaflutole may be monitored for formation of RPA 202248 when maintained under the same storage conditions as the spiking solutions and standards used.

4 Equipment and supplies

Cartridges, Spe-ed SPE, RP-102 resin (200 mg mL^{-1}) (Applied Separations, Cat. No. 4208; *no substitute*)
Column, HPLC, Columbus C_8, 50 mm × 2-mm i.d., 5 mm, 100-Å pore size (Phenomenex, Cat. No. 00B-4187-B0; *no substitute*)

Pre-column HPLC filter, ultra-low dead volume, 0.5-μm frit (Upchurch, A-318)
Cartridge adapters, SPE (University Research Glass, Cat. No. URG-2440-SPECA)
Stopcocks (plastic), Luer Lock (Varian, 1213-1005)
Zymark Benchmate Series I or II
Zymark EasyFill sample collection module
0.8% formic acid in water, pH 2.1
Water–acetonitrile (9 : 1, v/v, 0.8% formic acid, pH 2.1)
Water–acetonitrile (7 : 3, v/v)
Acetonitrile–methanol (1 : 1, v/v)
1.5% acetic acid in water

Note: all reusable glassware (except volumetric pipettes) should be baked in a muffle oven at 450 °C for at least 2 h to remove possible contamination before use.

5 Sampling and preparation

No specific sample processing or preparation is needed for this method. As is standard practice, water should be warmed to room temperature before sampling and analysis.

6 Extraction procedure

Two different extraction procedures were developed, a manual and an automated method. A work flow diagram of this residue analytical method is presented in Figure 1.

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6.1 Manual procedure

Acidify a 10-g water sample with 60 μL of formic acid. The sample may be stored in a refrigerator until needed. Add the appropriate amount of $^{13}C_6$-labeled internal standards. (An amount of 0.25 ng of $^{13}C_6$-labeled internal standard in a 10-g sample produces a concentration of 0.1 ng mL^{-1} in the final extract of about 2.5 mL; 0.1 ng mL^{-1} is the same internal standard concentration as is in the calibration standards.)

Condition an RP-102 SPE cartridge with 4 mL of acetonitrile–methanol (1 : 1, v/v) followed by 4 mL of HPLC-grade water (2 mL min^{-1}, do not allow the cartridge to dry).

Using an SPE reservoir, pass the entire sample through the conditioned cartridge at a rate of about one drop every 2 s.

Wash the cartridge with 1.0 mL of a solution of 0.8% formic acid in water followed by 1.0 mL of acetonitrile–water (3 : 7, v/v, at a rate of approximately one drop every 2 s). Do not allow the cartridge to dry out until the end of the washing step. Then dry the cartridge for 2 min using vacuum or positive nitrogen pressure.

Extract the analytes and internal standards from the cartridge by adding 1.0 mL of acetonitrile–methanol (1 : 1, v/v) to the cartridge followed by application of positive

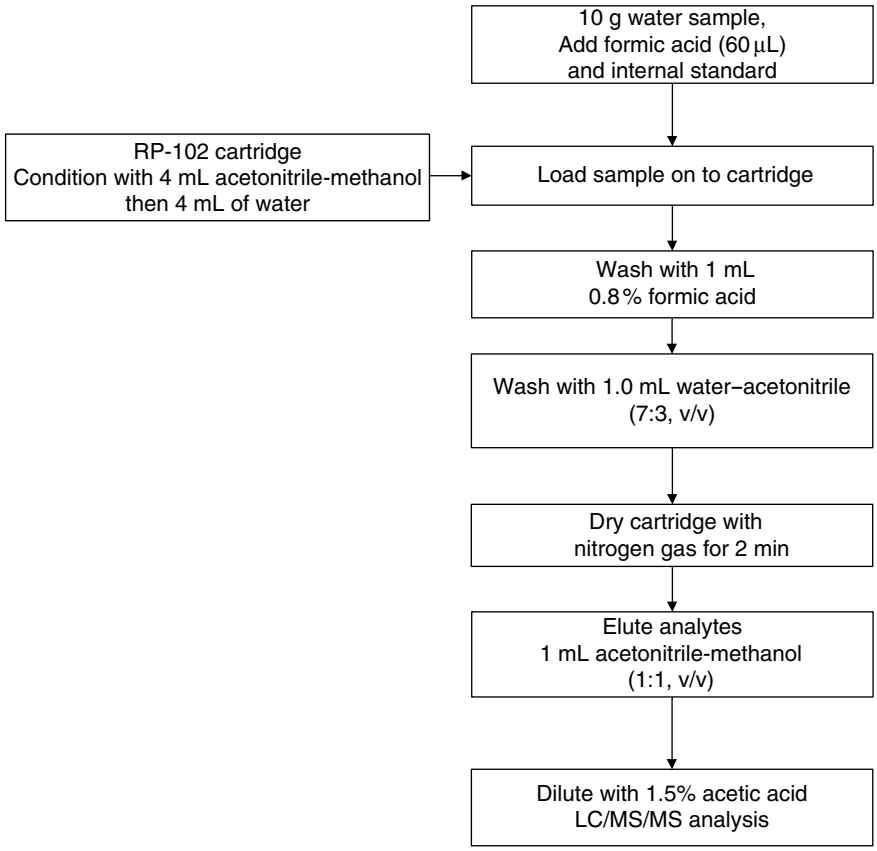


Figure 1 Flow diagram of method

pressure to push the solvent on to the cartridge. Take precautions to ensure that no eluent is lost. Positive pressure can be applied via a hand-held nitrogen line.

Vent the pressure and allow the cartridge to soak for 1–2 min. Reapply pressure and elute all solvent (1 drop s^{-1}) into an appropriately sized volumetric flask or chromatography vial.

Dilute the extract with 1.5% acetic acid in water and mix completely. Suggested final dilution volumes are 2.5 mL for samples containing expected residues near the limit of quantitation (LOQ) level of 10 ng L^{-1}. The extracts are placed in vials for LC/MS/MS analysis.

6.2 Automated procedure using a Zymark Benchmate Workstation with EasyFill module

An approximately 10-g water sample is acidified with 60 µL of formic acid and placed in a test-tube on the Benchmate Workstation along with up to 50 samples in total including any spiked quality control samples.

The Benchmate program is started. After unattended operation, the vials are removed from the EasyFill module and placed on the LC/MS/MS autosampler tray for analysis. Each Benchmate Workstation will process up to 50 samples in less than a 24-h period.

Below is a list of the automated steps performed by the Benchmate:

Step 0	Approximately 10-g sample is weighed accurately by the Benchmate balance.
Step 1	Adds 0.1 mL of internal standard (2 ng mL^{-1} mixture) per 10 g of tube contents.
Step 2	Mixes by cycling 10 mL in the tube five times.
Step 3	Conditions column with 4 mL of acetonitrile–water (1 : 1, v/v).
Step 4	Conditions column with 4 mL of HPLC-grade water.
Step 5	Loads sample on to column.
Step 6	Rinses column with 1.0 mL of 0.8% formic acid.
Step 7	Rinses column with 1.0 mL of acetonitrile–water (3 : 7, v/v).
Step 8	Dries column with gas for 120 s.
Step 9	Collects 1.1-mL fraction in next tube using acetonitrile–water (1 : 1, v/v).
Step 10	Adds 1.5 mL of 1.5% acetic acid.
Step 11	Mixes by cycling 2.5 mL in the tube five times.
Step 12	Washes EasyFill transfer line with 1 mL of acetonitrile–water (1 : 1, v/v).
Step 13	Washes EasyFill transfer line with 1 mL of 0.8% formic acid.
Step 14	Washes EasyFill transfer line with 1 mL of sample.
Step 15	Transfers 1.5 mL of sample into EasyFill vial.
Step 16	Washes EasyFill transfer line with 1 mL of acetonitrile–water (1 : 1, v/v).
Step 17	Washes syringe with 10 mL of acetonitrile–water (1 : 1, v/v).
Step 18	Washes syringe with 10 mL of 0.8% formic acid.
Step 19	End.

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Note that the indicated Benchmate parameters are guidelines and should be optimized for the instrument used. Instrument parameters may be adjusted to improve sample analysis.

6.2.1 Additional Benchmate settings

Flow rates	
Aspirate	1.00 mL s^{-1}
Dispense	1.00 mL s^{-1}
Internal standard	0.10 mL s^{-1}
Mix	1.25 mL s^{-1}
Air push	0.25 mL s^{-1}
SPE parameters	
Condition flow	0.20 mL s^{-1}
Load flow	0.08 mL s^{-1}
Rinse flow	0.20 mL s^{-1}
Elute flow	0.02 mL s^{-1}
Push delay	5 s
Air factor	1.5

7 Determination by LC/MS/MS

Instrument	Perkin-Elmer Sciex API 3000 LC/MS/MS system Perkin-Elmer Sciex Turbo IonSpray electrospray interface Shimadzu LC-10AD VP HPLC Pumps (2) with 250-μL high-pressure mixer and SCL-10A VP pump controller Gilson 215 autosampler
Ionization	Electrospray (Turbo IonSpray), negative ion mode
MS mode	MS/MS with multiple reaction monitoring (MRM)
IonSpray voltage	−4500 V
Orifice/ring voltage	Period 1: −59/−290 V Period 2: −31/−190 V
Nebulizer setting	15 (air)
Curtain gas setting	9 (nitrogen)
Turbo IonSpray settings	Heated air at 8.5 L min^{-1}, 500 °C
Collision gas setting	8 (nitrogen)
Collision energy (R02–Q0)	Period 1: (36 − 10) V = 26 V Period 2: (50 − 10) V = 40 V

Mass transitions (dwell times in milliseconds)

Period 1		
RPA 203328	267/159	(375 ms)
$^{13}C_6$-RPA 203328	273/165	(275 ms)
Period 2		
RPA 202248 and IFT	358/79	(375 ms)
$^{13}C_6$-RPA 202248	364/79	(275 ms)
$^{13}C_6$-IFT	364/79	(275 ms)

Column Phenomenex, Columbus C_8, 50 mm × 2.0-mm i.d., 5-μm particle size, 100-Å pore size

Notes:
1. Other brands tested did not retain RPA 202248.
2. The column needs to be reconditioned after about 12 h of use or whenever the RPA 202248 peak has shifted to a retention time greater than about 6 min. To recondition, the column should be flushed with 100% acetonitrile for 15 min and then stored in that solvent for about 8 h before re-use. Storing columns in mobile phase will result in an extremely long retention time and a tailing peak for RPA 202248. Just before use the columns will have to be conditioned with the mobile phase for 5–30 min or until the RPA 202248 peak is fully separated from the isoxaflutole peak.

Mobile phase flow rate	0.400 mL min^{-1}, no split
Mobile phase	Acetonitrile–1.5% acetic acid in HPLC-grade water (48 : 52, v/v)
Injection volume	95 μL
Retention times	RPA 203328 1.3 min Isoxaflutole 2.3 min RPA 202248 3.5–4 min These retention times may vary from system to system.

An example chromatogram is shown in Figure 2.

8 Evaluation

8.1 Performance criteria

First criterion

Inject a calibration standard solution corresponding to a level at or below the estimated LOQ and obtain a signal-to-noise ratio of at least 9 : 1.

If this criterion cannot be met, optimize the instrument operating parameters or change the instrument method parameters such as injection size until a signal-to-noise ratio of 9:1 is obtained.

If this criterion still cannot be met by changing the operating parameters, run higher level standards until a signal-to-noise ratio of 9 : 1 is obtained. This will require adjusting the method final sample dilution such that this standard level corresponds to the required LOQ.

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Second criterion

Run a set of standards of four or more concentration levels covering the expected range of residues. Generate a calibration curve for each analyte and obtain a linear regression with a correlation coefficient of at least 0.90 for each analyte. Do not use any sample run data if the combined regression for the standards run immediately before, during and after the samples does not meet this criterion.

8.2 Recoveries, limit of detection and limit of quantifiation

Method validation determined the limit of detection (LOD) to be 1 ng L^{-1} (ppt) for isoxaflutole, 1 ng L^{-1} for RPA 202248 and 3 ng L^{-1} for RPA 203328. However, after experience with a number of surface waters with high levels of matrix components, the method LOD was increased to 3 ng L^{-1} for all three analytes. RPA 202248 also proved to be particularly sticky and prone to carry over. Over time, this produced a background level, which also prevented determinations below the 3 ng L^{-1} method LOD.

8.3 Calculation

Generate calibration curves for isoxaflutole, RPA 202248 and RPA 203328. After the instrument performance criteria are met, a minimum of four standards over a range

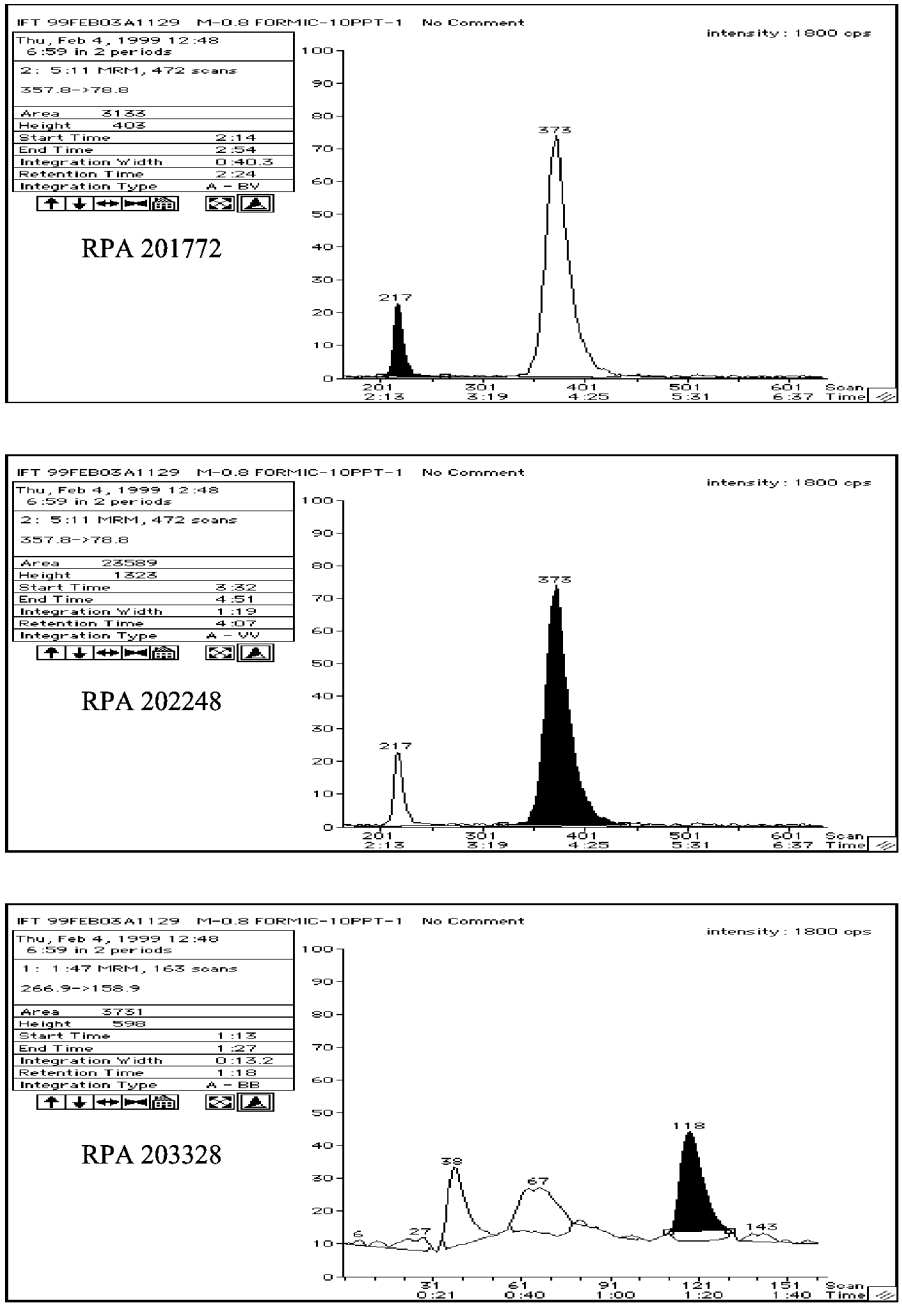


Figure 2 Surface water (1826 Ferriday, LA), fortified with 10 ng L^{-1} (ppt) isoxaflutole, RPA 202248 and RPA 203328

Table 1 Groundwater accuracy data

Procedure	Sample ID	Fortification level (ng L^{-1})	Accuracy (%)		
			RPA 203328	RPA 202248	Isoxaflutole
Automation	324AP2-1	0	ND	ND	ND
Automation	324AP2-2	10	97	100	95
Automation	324AP2-3	10	105	105	100
Automation	324AP2-6	100	98	100	103
Automation	324AP2-7	100	98	100	113

ND = not detected

of concentration levels should be included with a set of samples. Standards should be interspersed with samples or bracket samples to compensate for any minor change in instrument response. Samples should be diluted such that any peak areas or heights of the internal standards are approximately equal (±60%) to the internal standard peak responses in the calibration standards.

Linear regression coefficients should be calculated for the ratio of analyte to internal standard area or height plotted versus the ratio of analyte to internal standard concentration in the calibration standards. The data from the analytical standards should then be fitted to the linear model

$$y = A + Bx$$

where x = ratio of concentration to internal standard (IS) (^{13}C) concentration and y = response ratio = response (area)/IS response (area).

The equation to be used to estimate the residues in the samples is

$$E = \frac{(y - A)}{B} \times D \times f$$

where

y = ratio of analyte response (area or height) to internal standard response (area or height)
A = intercept from linear regression analysis
B = slope from linear regression analysis (area ratio per concentration ratio)

Table 2 Surface water accuracy data

Procedure	Sample ID	Fortification level (ng L^{-1})	Accuracy (%)		
			RPA 203328	RPA 202248	Isoxaflutole
Automation	3181676-BM1	0	ND	ND	ND
Automation	3181676-BM2	10	98	100	88
Automation	4021676-4BM	100	104	98	94
Manual	329-1826-1	0	ND	ND	ND
Manual	329-1826-2	10	93	100	110
Manual	329-1826-5	100	98	95	110

ND = not detected

D = ng L^{-1} internal standard in the starting sample = $[(I/d) \times c]/S$
I = weight of internal standard added to the sample (g)
d = density of internal standard solution (0.97)
c = concentration of internal standard solution (ng mL^{-1})
S = weight of starting sample (g)
f = dilution factor
E = concentration of analyte in sample in parts per billion (ppb or ng mL^{-1})

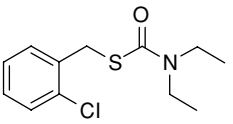
Recovery data from ground and surface water are summarized in Tables 1 and 2.

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Bayer CropScience, Research Triangle Park, NC, USA
Craig A. Smitley
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Sabrina X. Zhao
Pfizer Inc., Groton, CT, USA

Orbencarb

Materials to be analyzed	Wheat, soybean, potato and soil
Instrumentation	Gas-chromatographic determination

1 Introduction

Chemical name (IUPAC)	(*S*)-2-Chlorobenzyl diethylthiocarbamate
Structural formula	
Empirical formula	$C_{12}H_{16}ClNOS$
Molar mass	257.8
Melting point	9.0 °C
Boiling point	158 °C at 1 mmHg
Vapor pressure	12.4 mPa (20 °C)
Solubility	Water 24 mg L^{-1} (20–27 °C) Very soluble in organic solvents, e.g. acetone, xylene, n-hexane, ethanol and benzene, all >1 kg L^{-1} (room temperature)
Stability	Stable to hydrolysis for 60 days at pH 5–9 (20 °C)
Use pattern	Orbencarb is a systemic pre-emergence herbicide to control most annual grasses and broad-leaved weeds
Regulatory position	The definition of residues includes orbencarb and its metabolites, methyl 2-chlorobenzylsulfone (**I**) and 2-chlorobenzoic acid (**II**)

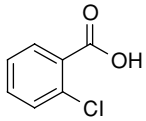
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2 Outline of method

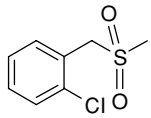
The determination of orbencarb in crops and soil is conducted by simultaneous analysis with its metabolites. In crops, orbencarb, **I** and **II** are extracted with acidic

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acetone by ultrasonication and transferred into an ethyl acetate–hexane mixture. After separating **II** with an alkaline aqueous solution, orbencarb and **I** remain in the organic solvent layer. Residues are purified by silica gel column chromatography and quantified by gas chromatography/electron capture detection (GC/ECD). Orbencarb and **I** in soil are extracted by refluxing with water containing methanol, transferred into dichloromethane, purified by silica gel column chromatography and quantified by gas chromatography/flame photometric detection (GC/FPD).



(I)



(II)

3 Equipment

Crusher (coffee-mill)
Homogenizer (Polytron mixer)
Ultrasonic cleaner: UC-6100, 600 W, 28 kHz (Sharp)
Round-bottom flasks: 200-, 300-, and 500-mL
Conical beaker: 500-mL
Buchner funnel: 10-cm i.d.
Separatory funnels: 200-, 300-, and 500-mL
Glass funnel
Condenser
Glass chromatography column: 400 × 15-mm i.d. with a stopcock
Column preparation: For orbencarb and **I**, a silica gel column is prepared by packing a slurry of silica gel (10 g) in dichloromethane–n-hexane (1 : 1, v/v) into a glass chromatography column. About a 1-cm layer of anhydrous sodium sulfate is placed above and below the silica gel bed. For **II**, a silica gel column is prepared by packing a slurry of silica gel (10 g) in dichloromethane–n-hexane (1 : 2, v/v) into a glass chromatography column. About a 1-cm layer of anhydrous sodium sulfate is placed above and below the silica gel bed
Rotary vacuum evaporator, 40 °C bath temperature

4 Reagents

Acetone, acetonitrile, dichloromethane, ethyl acetate, n-hexane and methanol: pesticide residue analysis grade
Hydrochloric acid, sodium chloride and sodium hydroxide: special grade
Anhydrous sodium sulfate: special grade
Silica gel: Wakogel C-200 (Wako Pure Chemical Industries, Ltd)
pH test paper

Diazomethane: In a distillation flask equipped with an distillation funnel and a cooler, place a solution of 5 g of potassium hydroxide in 8 mL of water and 25 mL of ethanol. Warm the distillation flask to 65 °C in a water-bath. Add a solution of 21.5 g (0.1 mol) of *N*-methyl-*N*-nitroso-*p*-toluenesulfamide in 130 mL of diethyl ether through the instillation funnel in 5 min. If the distillation funnel becomes empty, pour 20 mL of diethyl ether into the funnel, and distill it gradually. Continue distillation until the distilled ether solution becomes colorless. About 3 g of diazomethane is contained in the whole resultant ether distillate. **Caution:** these procedures should be conducted in a laboratory hood

Orbencarb, methyl 2-chlorobenzylsulfone (**I**), 2-chlorobenzoic acid (**II**), methyl 2-chlorobenzoate: analytical standard materials (Ihara Chemical Industries Co., Ltd)

Orbencarb and **I** standard solution for gas chromatography: 1.0 μg mL^{-1} in acetone

Methyl 2-chlorobenzoate standard solution for gas chromatography: 0.1 μg mL^{-1} in n-hexane

5 Sample preparation

Wheat grains and soybeans are ground in a coffee mill. Potato is chopped finely with a cutter.

6 Procedure

6.1 Extraction

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6.1.1 Plant material

Weigh 50 g of the sample into a 500-mL conical beaker, add 150 mL of acetone, 50 mL of water and 2.5 mL of concentrated HCl and sonicate the mixture for 30 min. For soybean and potato, add 150 mL of acetone, 50 mL of water and 4 mL of concentrated HCl, homogenize the mixture with a Polytron and sonicate for 30 min.

Filter the mixture through a filter paper by suction and collect the filtrate in a 500-mL round-bottom flask. Wash the residue and the beaker with 100 mL of acetone and filter and collect the washings in the same manner. Concentrate the combined acetone extracts in the round-bottom flask to about 60 mL, using a rotary evaporator under reduced pressure at 40 °C.

6.1.2 Soil

Weigh 50 g (dry soil base) of the sample into a 500-mL round-bottom flask and add 120 mL of methanol and 40 mL of water. Attach a condenser to the flask and reflux at 75 °C for 1 h. Filter the mixture through a filter paper by suction and collect the filtrate in a 500-mL round-bottom flask. Wash the residue and the flask with 80 mL of methanol and filter and collect the washings in the same manner. Combine the filtrates in a 500-mL separatory funnel.

6.2 Cleanup

6.2.1 Plant material

Transfer the concentrated sample extract (as described in Section 6.1.1) into a 200-mL separatory funnel and shake twice with 100 mL of ethyl acetate–n-hexane (1 : 1, v/v) solvent mixture.

Combine the ethyl acetate–n-hexane layer in a 500-mL separatory funnel, add 70 mL of 0.9 M sodium hydroxide solution and 10 mL of saturated sodium chloride aqueous solution (pH 10 or higher), shake the mixture and collect the organic layer. Wash the residual alkaline aqueous layer with 30 mL of n-hexane and combine the n-hexane layer with the organic layer. Using this partitioning procedure, Orbencarb and **I** are partitioned into the organic layer and **II** is partitioned into the alkaline aqueous layer.

Dry the organic solvent layer through 80 g of anhydrous sodium sulfate on a glass funnel and collect the dried solution in a 300-mL round-bottom flask. Evaporate the solvent under reduced pressure. Dissolve the residue in 150 mL of n-hexane and transfer the solution into a 300-mL separatory funnel. Extract twice with 100 mL of acetonitrile. Combine the acetonitrile extracts in a 500-mL round-bottom flask and evaporate the solvent under reduced pressure. Dissolve the residue in a small amount of column-eluting solvent (dichloromethane–n-hexane, 1 : 1, v/v) and transfer the solution to the top of the silica gel column. After eluting the column with 60 mL of solvent of the same composition (discard), elute orbencarb and **I** with 150 mL of dichloromethane. Collect the eluate in a 300-mL flask and evaporate the solvent under reduced pressure. Dissolve the residue in an appropriate volume of acetone for analysis.

The analytical procedure for the alkaline aqueous layer containing **II** is as follows. After acidifying the solution (about pH 2) with 4 mL of concentrated HCl, extract twice with 100 mL of dichloromethane. Dry the dichloromethane extract with anhydrous sodium sulfate and collect the dried solution in a 300-mL round-bottom flask. Evaporate the solvent under reduced pressure. Dissolve the residue in a mixed solvent consisting of 4 mL of ethyl acetate, 0.5 mL of methanol and 30 μL of concentrated HCl. To this mixture, add 7 mL of diazomethane–diethyl ether solution and allow the mixture to stand at room temperature for 1 h. Concentrate the reaction mixture to 0.5 mL under reduced pressure and evaporate the solvent in a gentle stream of nitrogen. Dissolve the residue in a small volume of dichloromethane–n-hexane (1 : 2, v/v) and transfer the solution to the top of column. Elute with solvent of the same composition, discard 60 mL of the initial eluate and collect 100 mL of the subsequent eluate in a 200-mL round-bottom flask. Concentrate the eluate to 0.5 mL under reduced pressure, evaporate the solvent in a gentle stream of nitrogen and dissolve the residue in an appropriate volume of acetone for analysis.

6.2.2 Soil material

Add 250 mL of water and 10 mL of saturated sodium chloride solution to the sample extract (as described in Section 6.1.2) and extract twice with 150 mL of dichloromethane. Dry the dichloromethane extract with anhydrous sodium sulfate,

collect the dried solution in a 500-mL round-bottom flask and evaporate the solvent under reduced pressure. Dissolve the residue in 150 mL of n-hexane and transfer the solution into a 300-mL separatory funnel. Extract twice with 100 mL of acetonitrile. Collect the acetonitrile layer in a 300-mL round-bottom flask and evaporate the solvent under reduced pressure. Dissolve the residue in a small volume of dichloromethane–n-hexane (1 : 1, v/v). Thereafter, conduct the cleanup of the soil sample by silica gel column chromatography as described in Section 6.2.1 to prepare the sample for determination of orbencarb and **I**.

6.3 Gas-chromatographic determination

Inject an aliquot (V_i) of the solution derived from Section 6.2 (V_{End}) into the gas chromatograph.

Operating conditions for orbencarb and I

Gas chromatograph	Hitachi model 163
Column	Glass, 3-mm i.d. × 1.0 m, packed with 3% NPGS on Chromosorb W HP, 100–120 mesh
Column temperature	190 °C
Injection port temperature	230 °C
Detector	Flame photometric detector fitted with a 394-nm sulfur-specific filter
Detector temperature	240 °C
Gas flow rates	Nitrogen carrier gas, 40 mL min^{-1} Hydrogen, 35 mL min^{-1} Oxygen, 20 mL min^{-1}
Attenuation	128 × 1000
Injection volume	0.5–6 µL
Retention time	Orbencarb 3.2 min, **I** 3.8 min
Minimum detectable amount	0.5 ng

Operating conditions for methylated II

Gas chromatograph	Hitachi Model 163
Column	Glass, 3-mm i.d. × 1.0 m, packed with 3% PEGA on Chromosorb W HP, 100–120 mesh
Column temperature	200 °C
Injection port temperature	240 °C
Detector	Electron capture detector (^{63}Ni, 10 mCi, pulse interval 100 µs)
Detector temperature	240 °C
Gas flow rate	Nitrogen carrier gas, 50 mL min^{-1}
Attenuation	32 × 10
Injection volume	1–5 µL
Retention time	3.9 min
Minimum detectable amount	0.1 ng

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7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Construct a fresh calibration curve with orbencarb standard solutions for each set of analyses. Using log–log paper, plot the peak heights in millimeters against the injected amount of orbencarb in nanograms. Peak heights of orbencarb on the chromatogram of a sample extract were measured. Orbencarb was quantified by comparing the peak height with the calibration curve. If **I** and **II** are determined simultaneously, the respective calibration curves should be prepared.

7.2 Recoveries and limits of detection

The recoveries from untreated control samples fortified with orbencarb at 0.2 mg kg^{-1} for crops and 0.5 mg kg^{-1} for soils were 85–98 and 89–101%, respectively. The limit of detection was 0.005 mg kg^{-1} for crops and 0.01 mg kg^{-1} for soils. The recovery of **I** was 92–102% from 0.2 mg kg^{-1}-fortified crops and 95–98% from 0.5 mg kg^{-1}-fortified soils. The recovery of **II** was 68–79% from 0.2 mg kg^{-1}-fortified crops and 73–77% from 0.5 mg kg^{-1}-fortified soils. The limits of detection of **I** and **II** were the same as those of orbencarb.

7.3 Calculation of residues

The residue R, expressed in mg kg^{-1} orbencarb, **I** or **II**, is calculated from the following equation:

$$R = (W_A \times V_{End})/(V_i \times G) \times F$$

where

G = sample weight (g)
V_{End} = terminal volume of sample solution from Section 6.2 (mL)
V_i = portion of volume of V_{End} injected into the gas chromatograph (μL)
W_A = amount of orbencarb for V_i read from the calibration curve (ng)
F = 1 for orbencarb and **I** and 0.92 for **II** (factor for conversion of methylated **II** to **II**)

8 Important points

8.1 Liquid–liquid partition

The partition rates of orbencarb and **I** in aqueous solutions (pH 2–12) into ethyl acetate–n-hexane (1 : 1, v/v) were as high as 87–90%, and the partition rate did not differ depending on the pH of the liquid. On the other hand, the recovery of **II** from aqueous solutions into ethyl acetate–n-hexane (1 : 1, v/v) was 85% at pH 2, 34% at

pH 7 and <3% at pH 12. Therefore, when orbencarb, **I** and **II** are separated by the partition procedures under alkaline conditions, the recovery of **II** will decrease unless the liquid is sufficiently alkaline. Since the property of the liquid extract of the sample is slightly different according to the type of analytical sample, it should be confirmed with a pH test paper that the pH of the aqueous layer is more than 10. Since the partition rate from n-hexane into acetonitrile is 99% for **I** and 75% for orbencarb, it is necessary to carry out the extraction twice to achieve a higher recovery of orbencarb.

8.2 Cleanup

Activated carbon column chromatography (1 g of Dalco G60 and 5 g of Avicel cellulose mixture): **I** and orbencarb elute into 0–50 mL and 100–300 mL of acetone eluate, respectively; a recovery of 95% or higher for both analytes is achieved for column cleanup, but it takes 2–3 h to complete.

8.3 Evaporation

Since methylated **II** is highly volatile, evaporation of the solvent to dryness should be avoided.

8.4 Detection

For gas-chromatographic analysis, orbencarb and **I** are detected with good peak shapes with a column using as liquid phases silicone SE-30, Thermon-3000, FFAP and PEGA, but NPGS is superior for separation from impurities.

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*8.5 Determination of Metabolite **II** in soil*

The recovery is low on extracting **II** from soil by refluxing with water containing methanol. Metabolite **II** in soil can be extracted by sonication with alkaline methanol. Using this alkaline extraction procedure, another metabolite, 2-chlorobenzylsulfonic acid, is also extracted simultaneously.[1]

Reference

1. M. Ikeda, Y. Asano, and K. Ishikawa, 'Analytical methods for chlorobenzylsulfonic acid in crops, soils and water,' in "Abstracts of the 13th Annual Meeting of the Pesticide Science Society of Japan," p. 145 (1988).

Further reading

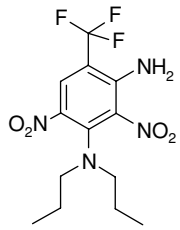
M. Ikeda, T. Unai, and C. Tomizawa, *J. Pestic. Sci.,* **11**, 85 (1986).
M. Ikeda, T. Unai, and C. Tomizawa, *J. Pestic. Sci.,* **11**, 97 (1986).

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Prodiamine

Materials to be analyzed	Air, soil, and water
Instrumentation	Gas chromatography with electron capture or mass-selective detection

1 Introduction

Chemical name (IUPAC)	5-Dipropylamino-α,α,α-trifluoro-4,6-dinitro-*o*-toluidine
Structural formula	
Empirical formula	$C_{13}H_{17}F_3N_4O_4$
Molar mass	350.29
Melting point	122.5–124.0 °C
Vapor pressure	2.5×10^{-8} mbar at 25 °C
Solubility (25 °C)	Water 13 μg L^{-1}, acetone 226 g L^{-1}, n-octanol 9.62 g L^{-1}
Solubility (20 °C)	Dimethylformamide 321 g L^{-1}, xylene 35.4 g L^{-1}, heptane 1.0 g L^{-1} at 20 °C
Other properties	Munsell color yellow–orange powdered solid; density 1.41 g mL^{-1}, log K_{ow} 4.1, thermally stable
Use pattern	Prodiamine is used in alfalfa, cotton, soybeans, vines, nuts, and turf as a pre-plant and post-emergence grass and broad-leaved herbicide
Regulatory position	The definition of residue is for the parent, prodiamine, only

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2 Outline of method

Prodiamine is extracted from air by passing a known volume through Chromosorb collection tubes at a predetermined rate and time. The adsorption tubes are extracted with toluene followed by analysis using gas chromatography/electron capture detection (GC/ECD). Soil is extracted with methanol via mechanical shaking and partitioned into dichloromethane. Further cleanup is provided using silica gel followed by analysis using either GC/ECD or gas chromatography/mass spectrometry (GC/MS). Prodiamine is extracted from water by partitioning into dichloromethane followed by cleanup on a Florisil column via elution using pentane–diethyl ether (19 : 1, v/v) solvent mixture.

3 Apparatus

Air sampling tubes, Chromosorb 102 (Cat. No. 226-49-20-102) from SKC, Inc. (Eight-Four, PA, USA)
Bottles, 2-oz, screw-cap with Polyscal liner
Bottles, screw-cap with polyseal, 32- and 8-oz, amber-colored
Centrifuge, Model CS, International Equipment Co.
Chromatographic columns, 20-cm × 9-mm-i.d., with 50-mL reservoir
Dish, Pyrex
Distillation receiver tubes, 15-mL, graduated, with stoppers
Distillation receivers, 15-mL Kuderna-Danish concentrator, 125-mL
Mass flow meter, Kurz Model 541, Kurz Instruments, Inc. (Carmel Valley, CA, USA)
N-Evap with 40 °C water-bath
Oven, 150 °C
Pump, vacuum, Gilian Model HFS 1137
Platform shaker
Separatory funnels, 500-mL
Vigreaux condenser
Water-bath, 60 °C

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4 Reagents

Dichloromethane, residue analysis grade
Diethyl ether containing 2% ethanol preservative, residue analysis grade
Florisil, 60–100-mesh
Methanol, residue analysis grade
Pentane, residue analysis grade
Silica gel 60, 70–200-mesh, EM reagents
Sodium chloride, reagent grade
Sodium sulfate, anhydrous, granular, reagent grade
Toluene, reagent grade
Toluene, residue analysis grade

5 Sample preparation

5.1 Air

Air samples are typically collected by passing a known volume of air for a specific time period through Chromosorb 102 air sampling tubes (e.g. 1.5 L min^{-1} for 2 h) using a Gilian or similar pump and a flow meter. For extraction, the contents of the tube are emptied into a 15-mL distillation receiver and extracted with 10 mL of toluene briefly at 5-min intervals for 15 min. After centrifugation, a portion of the toluene is removed and analyzed using GC/ECD.

5.2 Soil

Shake 20 g of soil for 30 min with 200 mL of methanol in an 8-oz jar on a mechanical, reciprocal shaker. Centrifuge for 30 min at 550 *g* or until the supernatant is clear. Decant the supernatant and transfer 50 mL (equivalent to 5 g) to a 500-mL separatory funnel containing 250 mL of 5% sodium chloride solution and 25 mL of dichloromethane. Shake for 1 min and, after phase separation, drain the dichloromethane fraction through a bed of anhydrous sodium sulfate and collect in a Kuderna-Danish (K-D) evaporator. Repeat the partitioning steps twice more, each time using 25 mL of dichloromethane following the drying procedure described above and collecting in the same evaporator. Wash the sodium sulfate bed three times with 5–10 mL of dichloromethane and collect the washings in the evaporator. Add 1 mL of hexane to the dried dichloromethane fraction, attach a Vigreaux condenser, and evaporate to near dryness in a 60 °C water-bath. Add 25 mL of pentane and again reduce to near dryness to remove all traces of dichloromethane. Prepare a silica gel column by slowly adding 20 g of 3% water-deactivated silica gel to a 250-mL separatory funnel containing 70 mL of pentane–diethyl ether (9 : 1, v/v). Shake well and drain the suspension into a chromatographic column plugged with glass-wool. After the silica gel has settled, cap with 1 cm of anhydrous sodium sulfate and drain the solvent just to the top of the sodium sulfate layer. Dissolve the residue in the K-D evaporator using 5 mL of pentane–diethyl ether (9 : 1, v/v) and transfer to the silica gel column. Rinse the K-D evaporator twice, each time with 5 mL of pentane–diethyl ether (9 : 1, v/v) and transfer to the column. Wash the column with 70 mL of pentane–diethyl ether (9 : 1, v/v) and discard. Elute the analyte with 75 mL of pentane–diethyl ether (9 : 1, v/v) and collect in a K-D evaporator. Attach a Vigreaux condenser and reduce to near dryness in a 60 °C water-bath. Evaporate the last traces of solvent with a gentle stream of dry nitrogen gas and reconstitute the residue using 5 mL of toluene.

5.3 Water

Water is extracted by partitioning 100 mL of sample three times with 25-mL portions of dichloromethane. The organic fractions are pooled, dried through anhydrous sodium sulfate, and evaporated to dryness using a gentle stream of dry nitrogen gas.

The residue is reconstituted to 1 mL in pentane–diethyl ether (19 : 1, v/v). This fraction is loaded on to a 15-cm bed of Florisil previously capped with 1 cm of anhydrous sodium sulfate and with pre-wetting of the column bed with pentane–diethyl ether (19 : 1, v/v). The vessel with the previously reconstituted fraction is rinsed twice each time with 1 mL of pentane–diethyl ether (19 : 1, v/v) and the rinsates are added to the Florisil column. The column is washed with 10 mL of pentane–diethyl ether (19 : 1, v/v) and this fraction is discarded. The analyte is eluted with 25 mL of pentane–diethyl ether (19 : 1, v/v) and the eluate is collected in a K-D evaporator. A Vigreaux condenser is fitted and the organic fraction is evaporated to near dryness (the last traces of diethyl ether and pentane are removed using a gentle stream of dry nitrogen gas). The residue is reconstituted to 5 mL using toluene.

6 Instrumentation

The following instrumental conditions have been shown to be suitable for the analysis of prodiamine. Other operating parameters may be employed provided that prodiamine is separated from sample interferences and the response is linear over the range of interest.

Operating conditions

Gas chromatograph	Hewlett-Packard, Model 5710A with ^{63}Ni electron-capture detector
Column	Glass, Pyrex, 2 m × 2-mm-i.d. with 3% QF-1 on 80–100-mesh Gas Chrom Q
Column temperature	200 °C
Injection port temperature	250 °C
Detector	Electron capture detector, temperature 300 °C
Gas flow rates	5% methane in argon, 30 mL min^{-1}
Injection volume	2–8 µL for packed column and 2 µL for capillary column
Retention time for prodiamine	4.8 min
Gas chromatograph	Tracor MT-220 with ^{63}Ni electron capture detector
Column	Glass, Pyrex, 2 m × 2-mm-i.d. with 3% SE-30 on 80–100-mesh Gas Chrom Q
Column temperature	190 °C
Injection port temperature	210 °C
Detector	Electron capture detector, temperature 320 °C
Gas flow rates	5% methane in argon, 45 mL min^{-1}
Scavenger	5% methane in argon, 135 mL min^{-1}
Injection volume	2–8 µL for packed column and 2 µL for capillary column
Retention time for prodiamine	2.3 min
Gas chromatograph	Hewlett-Packard, Model 5880A with Model 5870 mass-selective detector

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Column	12.5 m × 0.2-mm i.d. fused silica with 50% phenyl–methyl silicone bonded phase, 0.33-μm film thickness
Column temperature	100 °C for 5 min, increased at 30 °C min^{-1} to 190 °C (held for 5 min)
Injection port temperature	250 °C
Detector	Mass-selective detector, temperature 300 °C
Interface	190 °C
Gas flow rates	He, inlet pressure 15 psi
Injection volume	2 μL for capillary column
Retention time for prodiamine	5.9 min
Selected ion monitoring	m/z 279, 321 (target ion), and 333

7 Evaluation

7.1 Method

Quantitation was performed in all cases using the external calibration method. A series of standards were injected and the responses plotted against their known concentrations. Peak responses in samples were compared with the calibration plots to obtain the amount found (nanograms). A fresh calibration plot was generated with each analytical set of samples.

7.2 Recoveries, limit of detection (LOD) and limit of quantitation (LOQ)

The recoveries from air were evaluated by spiking known quantities into Pyrex tubes and then passing air through the tube and collection on two Chromosorb tubes in series. Recoveries in the first Chromosorb tube were typically >97%. The recoveries obtained from fortified Chromosorb tubes ranged from 89 to 101%. The LOD was 0.5 ng L^{-1} when air was sampled at 1.5 L min^{-1} for 2 h.

The recoveries for soil ranged from 91 to 96% at the LOQ of the method (0.10 mg kg^{-1}).

The recoveries for water at the LOQ (10 μg L^{-1}) and 100 μg L^{-1} fortification levels were 95 and 91%, respectively. The LOD was 1 μg L^{-1}.

7.3 Calculation of residues

Air

$$C(\mu g\,L^{-1}) = WV/FTV_i \quad \text{or} \quad C(\mu g\,L^{-1}) = \frac{[C(\mu g\,L^{-1}) \times 24 \times 4] + 5}{MW}$$

where $C(\mu g\,L^{-1})$ = concentration of prodiamine in air ($\mu g\,L^{-1}$), W = weight of prodiamine in injected aliquot (ng) (from calibration curve), V = sample extract volume (mL), F = sampler flow rate ($L\,min^{-1}$), T = sampling time (min), V_i = volume of injected aliquot (μL), and MW = molecular weight of prodiamine (350.3)

Soil and water

$$R = ng \times V_S / V_i W$$

where R = concentration of prodiamine ($mg\,kg^{-1}$), ng = weight of prodiamine in injection (ng) (from calibration curve), V_S = volume of final sample extract (mL), V_i = volume of injected aliquot (μL), and W = weight of sample used for analysis (g)

8 Important point

The use of GC/MS is preferred owing to the confirmatory nature of the analysis.

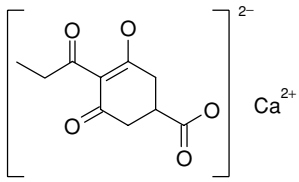
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Prohexadione-calcium

Materials to be analyzed	Rice (grain, straw), wheat (grain), barley (grain), strawberry and soil
Instrumentation	High-performance liquid chromatographic determination

1 Introduction

Chemical name (IUPAC)	Calcium 3-oxido-5-oxo-4-propionylcyclohex-3-ene-carboxylate
Structural formula	
Empirical formula	$C_{10}H_{10}CaO_5$
Molar mass	250.3
Melting point	$>360\,^{\circ}C$
Vapor pressure	0.0133 mPa at 20 °C
Solubility	Water 174 mg L^{-1} (20 °C), methanol 1.11 mg L^{-1} (20 °C), acetone 0.038 mg L^{-1} (20 °C).
Stability	In water; DT_{50} 5 days at pH 5 and 83 days at pH 9 (20 °C). Stable to heat (200 °C). Under sunlight in water, DT_{50} 4 days
Use pattern	Prohexadione-calcium, a plant growth regulator and retardant, is used as an anti-lodging agent in small grain cereals and it could also be used as a growth retardant in turf, peanuts, flowers and to inhibit new twig elongation of fruit trees.
Regulatory position	The residue definition is for the parent, prohexadione-calcium, only.

2 Outline of method

Prohexadione-calcium in the samples is extracted with acidic acetone by shaking (extracted as the free acid, prohexadione). The extract is purified by a series of

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procedures of liquid–liquid partition, ion-exchange column chromatography, methyl esterification (production of methyl ester of prohexadione) and reversed-phase column chromatography depending on the interfering materials in the analytical samples. Prohexadione-calcium is determined by high-performance liquid chromatography (HPCL) with ultraviolet (UV) detection (274 nm).

3 Apparatus

Mill (coffee-mill type)
Grinder (cutting mills, Willey type)
Blender (kitchen type)
Round-bottom flasks, 500-, 300-, 200- and 100-mL
Separatory funnels, 200- and 50-mL
Stoppered test-tube, 30-mL
Glass funnels, 10- and 4.5-cm i.d.
Condenser
Glass chromatography column (reversed-phase silica gel, 1.5-cm i.d. × 40 cm, DEAE ion exchanger, 1.0-cm i.d. × 30 cm)
Reversed-phase silica gel column: Place a cotton wool plug at the bottom of a glass chromatography column. Pack 5 g of reversed-phase silica gel slurried with a solvent mixture of n-hexane–benzene–methanol (80 : 20 : 0.4, v/v/v) into the glass column. Place an anhydrous sodium sulfate layer about 1-cm thick above and below the silica gel bed
Bell jar-type filtering apparatus
Buchner funnel, 11-cm i.d.
Rotary vacuum evaporator, 40 °C bath temperature
Dry-block bath, electrically heated, temperature 75 °C
Mechanical shaker (universal shaker)
Ultrasonic cleaner
High-performance liquid chromatograph equipped with a UV detector
Microsyringe, 25-μL

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4 Reagents

Acetone, acetonitrile, benzene, dichloromethane, n-hexane and methanol, pesticide residue analysis grade
Chloroform, sodium chloride, anhydrous sodium sulfate, sulfuric acid (97%), hydrochloric acid (36%), sodium bicarbonate, trifluoroacetic acid, tris(hydroxymethyl)aminomethane (Tris), special grade
Water, high-performance liquid chromatography grade
0.1 M Phosphate buffer solution (pH 7.0)
Reversed-phase silica gel, silica gel ODS-Q3, 75A, 30–50-μm (Wako Pure Chemical Industries, Ltd)
DEAE ion exchanger, Cellulofine A-200 (Wako Pure Chemical Industries, Ltd)
Filter paper, 11-cm i.d.
pH test paper

Prohexadione-calcium, analytical grade (Ihara Chemical Industries Co., Ltd)

Prohexadione-calcium standard solutions: 0.05, 0.2, 0.4, 0.6 and 0.8 mg L^{-1} in acetonitrile

Preparation of Tris–HCl buffer: Dissolve 60.5 g of Tris in 400 mL of distilled water, add concentrated hydrochloric acid to adjust to pH 7.7 and add distilled water to make exactly 500 mL to prepare a 1.0 M Tris–HCl buffer, which should be stored at 5 °C. Prepare 0.05 M and 0.01 M buffers by diluting with distilled water before use.

Preparation of ion-exchange column: *Preparation of ion-exchange resin.* To 500 mL of Cellulofine 200A (DEAE ion exchanger), add 500 mL of water, mix well and filter this mixture under reduced pressure. Wash the residue (ion-exchange resin) twice with 300 mL of 0.5 M Tris–HCl buffer (pH 7.7) and filter this mixture under reduced pressure. Swell the ion-exchange resin in 100 mL of the buffer for 1 h to activate the resin. Remove the buffer by filtration and swell the ion-exchange resin in 500 mL of 0.01 M Tris–HCl buffer (pH 7.7) for about 10 min. Remove the buffer by filtration, swell and wash the ion-exchange resin three times with 200 mL of the buffer for about 10 min each, and filter this mixture by suction. Swell the washed ion-exchange resin in 100 mL of 0.01 M Tris–HCl buffer (pH 7.7) and store as it is. *Preparation of column.* Plug the bottom of a glass chromatography column of 1 cm i.d. with absorbent cotton and pack the column with 5 mL of the prepared ion-exchange resin suspended in 0.01 M Tris–HCl buffer (pH 7.7) by the wet method. Wash the resin in the column with about 20 mL of the buffer before use.

Prohexadione-calcium standard solutions: Dissolve 10 mg of prohexadione-calcium in 100 mL of water to prepare a 100 mg L^{-1} solution. Transfer 100 μL of this solution into a 30-mL test-tube, evaporate water to dryness under reduced pressure and to methylate prohexadione-calcium according to Section 6.3. Dissolve the product in acetonitrile to prepare the 0.05, 0.2, 0.4, 0.6 and 0.8 mg L^{-1} acetonitrile solutions.

5 Sampling and sample preparation

Collect 1 kg each of rice grain, wheat grain and barley grain and grind them with a mill. Collect 1 kg of rice straw and grind it with a grinder. Collect 1 kg of strawberry and homogenize with a blender. Collect soil, from the top10-cm surface layer, homogenize and pass through a 5-mm sieve.

6 Procedure

6.1 Extraction

Weigh the samples [strawberry, rice grain, 25 g; wheat grain, barley grain, 10 g; rice straw, 5 g; soil (dry weight basis), 30 g] in round-bottom flasks of appropriate volumes (500- or 300-mL). For soil samples add 40 mL of 1 N sulfuric acid and 120 mL of acetone to the flask, and for other samples add 20 mL of 1 N sulfuric acid and 60 mL

of acetone to the flask. Shake the flask at room temperature for 30 min (prohexadione-calcium is extracted as the free acid, prohexadione). Filter the extract through a filter paper on a Buchner funnel with suction into a flask of an appropriate volume (500- or 300-mL). Wash the residue and the flask with 80 mL of acetone, and filter them in a similar manner. Combine the filtrates and concentrate under reduced pressure (to about 50 mL for soil and to about 20 mL for others).

Transfer the concentrate of soil sample extract into a 200-mL separatory funnel with a small volume of water. To the concentrate, add 1 mL of concentrated sulfuric acid and partition twice with 50 mL of n-hexane. Discard the n-hexane layer.

Transfer the concentrated crop sample extract (strawberries, rice grain, barley grain and rice straw) into a 50-mL separatory funnel with a small volume of water. Extract the solution three times with 10 mL of a chloroform–methanol (3 : 1, v/v). Dry the chloroform–methanol layer with a small amount (about 8 g) of anhydrous sodium sulfate on a glass funnel and transfer the dried solution to a 100-mL separatory funnel.

For wheat grain, extract the concentrate three times with 10 mL of chloroform–methanol (3 : 1, v/v). For the soil sample, extract the aqueous layer after washing with n-hexane twice with 60 mL of chloroform–methanol (3 : 1, v/v). Dry the chloroform–methanol layer with anhydrous sodium sulfate [for wheat grain, use a small amount (about 8 g) of anhydrous sodium sulfate] and collect the dried solution in a 200-mL round-bottom flask. Evaporate the solvent under reduced pressure and proceed to ion-exchange column chromatography.

Extract the chloroform–methanol layer from the strawberry, rice grain, barley grain and rice straw samples twice with 30 mL of 0.1 M phosphate buffer solution (pH 7.0). Since an emulsion is formed, the first extraction should be conducted with very gentle shaking. Centrifuge the extract at 2500 rpm for 10 min, when an emulsion is formed. Discard the chloroform–methanol layer.

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Combine the aqueous layers, add 3.5 mL of concentrated sulfuric acid and extract the solution twice with 60 mL of a mixture of chloroform and methanol. Dry the chloroform–methanol layer with anhydrous sodium sulfate and collect the dried solution in a 200-mL round-bottom flask. Evaporate the solvent under reduced pressure.

6.2 Ion-exchange column chromatography

To the flasks for the crop and soil samples (Section 6.1), add 2 mL of 0.01 M Tris–HCl buffer solution (pH 7.7) and 50 and 100 μL of 1 M Tris–HCl buffer solution for wheat grain, barley grain and rice straw, and for soil, respectively. Adjust the pH to about 7.7 (confirm the pH with a pH test paper using the sample of untreated area). Homogenize the residue with ultrasonication and transfer the homogenate to the top of an ion-exchange column. Wash the flask twice with 2 mL of 0.01 M Tris–HCl buffer solution and transfer the washings to the column. Elute the column with 40 mL of the same buffer solution. Discard this eluate.

Subsequently, elute the target substance with 50 mL of the same buffer solution containing 0.1 M sodium chloride. Transfer this eluate to a 200-mL separatory funnel,

add 1 mL of concentrated sulfuric acid to the solution and extract twice with 50 mL of chloroform–methanol (3 : 1, v/v). Dry the chloroform–methanol layer with anhydrous sodium sulfate and collect the dried solution in a 200-mL round-bottom flask. Evaporate the solvent to dryness under reduced pressure.

6.3 Methylation

Transfer crop and soil samples from Section 6.1 (strawberry and rice grain) and Section 6.2 with 5 mL of methanol to 30-mL test-tubes and add to each test-tube 0.05 mL of concentrated sulfuric acid. Attach a condenser and reflux the solution at 75 °C for 60 min to esterify prohexadione to its corresponding methyl ester. Cool the reaction mixture to room temperature, add 20 mL of water and extract the reaction solution twice with 20 mL of dichloromethane. Dry the dichloromethane layer with a small amount of anhydrous sodium sulfate and collect the dried solution in a 100-mL round-bottom flask. Evaporate the solvent under reduced pressure.

For soil samples, dissolve the residue in an appropriate volume of acetonitrile prior to HPLC analysis.

Dissolve the crop residue samples from above in 0.5 mL of dichloromethane and add 20 mL of n-hexane to the solution. Transfer the mixture to a 50-mL separatory funnel, add 20 mL of 0.2 M sodium bicarbonate solution and shake the funnel. Since an emulsion may be formed during shaking, initially shake the funnel very gently. Centrifuge at 2500 rpm for 10 min, if necessary. Collect the aqueous layer and discard the n-hexane layer.

Add 0.8 mL of concentrated sulfuric acid (pH 2–3) to the aqueous layer and extract twice with 20 mL of dichloromethane. Dry the dichloromethane layer with anhydrous sodium sulfate and collect the dried solution in a 100-mL round-bottom flask. Evaporate the solvent under reduced pressure.

For wheat grain, dissolve the residue in an appropriate volume of acetonitrile prior to HPLC analysis.

6.4 Reversed-phased silica gel column chromatography cleanup

Dissolve the crop residue samples from Section 6.3 in 0.5 mL of dichloromethane. Transfer this solution to the top of the reversed-phased silica gel column with 4.5 mL of n-hexane–benzene–methanol (80 : 20 : 0.4, v/v/v) and elute with the same solvent. Discard the first 70-mL of the eluate and collect the second 100-mL of eluate in a 200-mL round-bottom flask. Evaporate the solvent under reduced pressure.

Dissolve the residue in an appropriate volume of acetonitrile for HPLC analysis.

6.5 High-performance liquid chromatographic determination

Inject an aliquot (V_i) of the soil and crop samples into the HPLC system.

Operating conditions

Instrument	LC-10AD equipped with an SPD-10A UV spectrophotometric detector (Shimadzu Co., Ltd, Japan)
Column	CAPCELL PAK C_{18} SG 120 (Shiseido Co., Ltd, Japan), 4.6-mm i.d. × 250 mm; column temperature, ambient
Mobile phase	Acetonitrile–distilled water–trifluoroacetic acid (20 : 30 : 0.1, v/v/v)
Flow rate	1.0 mL min^{-1}
Wave length	274 nm
Attenuation	0.002 absorbance
Chart speed	10 mm min^{-1}
Injection volume	1–10 μL
Retention time	8.4 min
Minimum detectable amount	0.5 ng

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Construct a fresh calibration curve with prohexadione-calcium standard solutions for each set of analyses. Inject 10 μL of each prohexadione-calcium standard solution into the HPLC system. Using log–log paper, plot the peak heights in millimeters against the injected amount of prohexadione calcium in nanograms. Also inject 1–10-μL aliquots of the sample solutions. For the heights of the peaks obtained for these solutions, read the appropriate amounts of prohexadione-calcium from the calibration curve.

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7.2 Recoveries and limits of detection

The recoveries from control samples fortified with prohexadione-calcium at a level of 0.2 mg kg^{-1} were 75–80% for strawberry, 79–83% for rice grain, 79–89% for wheat grain, 92–105% for barley grain and 81–89% for soil. The recoveries from control samples fortified with prohexadione-calcium at a level of 0.5 mg kg^{-1} were 69–75% for rice straw. The limits of detection were 0.01 mg kg^{-1} for wheat grain and soil, 0.02 mg kg^{-1} for strawberry, rice grain and barley grain and 0.05 mg kg^{-1} for rice straw.

7.3 Calculation of residues

The residue R, expressed in mg kg^{-1} prohexadione-calcium, is calculated from the following equation:

$$R = (W_A \times V_{End})/(V_i \times G)$$

where

G = sample weight (g)
V_{End} = terminal volume of sample solution (mL)
V_i = portion of volume V_{End} injected into HPLC system (μL)
W_A = amount of prohexadione calcium for V_i read from calibration curve (ng)

8 Important points

1. Since prohexadione-calcium degrades rapidly in soil, soil samples should be analyzed or frozen immediately after sampling.[1]
2. The extraction method for prohexadione-calcium in soil was developed using alluvial soil and volcanic ash soil. Extraction by shaking the soil with a mixture of 1 N sulfuric acid–acetonitrile (1 : 3, v/v) and/or of 1 N sulfuric acid–acetone (1 : 3, v/v) showed an acceptable extraction recovery efficiency.
   Prohexadione in aqueous solution is not partitioned in n-hexane. More than 85% of prohexadione in solutions of pH 4 or lower is partitioned in ethyl acetate.
3. *Methyl esterification:* When prohexadione was treated with methanolic HCl (3%, w/v) or sulfuric acid–methanol (1%, v/v) under reflux at 75 °C for 60 min, or with BF_3–methanol (14%, w/v) under reflux at 75 °C for 30 min, the yield of the methyl ester of prohexadione was 95, 93 and 82%, respectively (prohexadione, 10 μg, volume 2 mL of methanolic HCl, 2 mL of sulfuric acid–methanol and 1 mL of BF_3–methanol). A solution of 1% (v/v) sulfuric acid in methanol was chosen for ease of preparation. Even if prohexadione was treated with 1% sulfuric acid in methanol at room temperature for 12 h, the yield of prohexadione methyl ester was not different from that under reflux conditions as described in Section 6.3. The conditions for methyl esterification in Section 6.3 were chosen because of shortening of the analysis time and the reproducibility of the reaction yield in residue analysis samples which could contain large quantities of contaminants.
   Since the methyl ester of prohexadione had lower polarity compared with prohexadione, an ODS column was very useful for purifying the sample.
   Since the methyl ester of prohexadione degrades rapidly in 1 M NaOH, it should not be handled under alkaline conditions.
4. Prohexadione-calcium degrades in aqueous sodium hypochlorite solution. Prohexadione-calcium at the level of 0.08 mg kg^{-1} in tap water degrades and disappears rapidly.[2] Degradation of prohexadione-calcium can be prevented by addition of ascorbic acid at about 1 mg kg^{-1} in tap water. Degradation products of prohexadione-calcium by aqueous chlorination are identified by mass spectrometry.
5. *Sample storage stability:* Prohexadione-calcium in strawberry, rice grain, rice straw, wheat grain and barley grain is stable at −20 °C for 40, 140, 60, 80 and 100 days, respectively. Approximately 88% of the applied prohexadione-calcium remained in soil when stored at −20 °C after 80 days.

References

1. A. Yagi, 'An example of the evaluation of the behavior in the environment of new agricultural chemicals,' in "Proceedings of the 12th Symposium on Environmental Science of Pesticides," pp. 19–25 (1994) (in Japanese).

2. M. Ikeda, Y. Asano, and Y. Yusa, 'Degradation of prohexadione-calcium by aqueous chlorination,' in "Abstracts of the 20th Annual Meeting of the Pesticide Science Society of Japan," p. 152 (1995).
3. A. Yagi, K. Mizuno, Y. Asano, and K. Ishikawa, 'Residue analytical method for the calcium salt of 3,5-dioxo-4-propionylcyclohexane-1-carboxylic acid (prohexadione-calcium) in crops,' in "Abstracts of the 16th Annual Meeting of the Pesticide Science Society of Japan," p. 114 (1991).

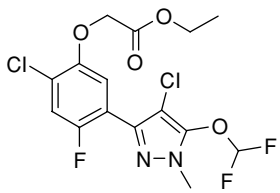
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Pyraflufen-ethyl

Materials to be analyzed	Plants (cereals, cotton, potato, citrus, apple, pear, peach, grape, persimmon, apricot, chestnut), soil and water
Instrumentation	Gas-chromatographic determination [mass spectrometric detection (MSD), flame thermionic detection (FTD) or nitrogen–phosphorus detection (NPD)] for plant materials, water and soil

1 Introduction

Chemical name (IUPAC)	Ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate
Structural formula	
Empirical formula	$C_{15}H_{13}Cl_2F_3N_2O_4$
Molar mass	413.2
Melting point	126–127 °C
Boiling point	Not measurable, decomposition above its melting point
Vapor pressure	1.6×10^{-8} Pa at 25 °C
Solubility	Water 0.082 mg L^{-1}, n-hexane 234 mg L^{-1}, methanol 7.39 g L^{-1}, 1,2-dichloroethane 100–111 g L^{-1}, acetone 167–182 g L^{-1}, *p*-xylene 41.7–43.5 g L^{-1}, ethyl acetate 105–111 g L^{-1} (all at 20 °C)
Stability	Rapidly degraded under alkaline conditions. DT_{50}: >120 h (pH 4 at 50 °C); 13 days (pH 7 at 25 °C); <2.4 h (pH 9 at 50 °C) Rapidly degraded under artificial light. DT_{50}: 30 h (pH 5 at 20 °C).
Other properties	Log $P_{ow} = 3.49$ by high-performance liquid chromatography (HPLC)

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Use pattern

Pyraflufen-ethyl was primarily developed as a cereal herbicide to control a broad spectrum of broadleaf weeds.[1] Pyraflufen-ethyl applied in early post-emergence at 12 g a.i. ha^{-1} provides excellent control of some important weeds such as *Anthemis arvensis*, *Lamium purpureum* and *Sinapis arvensis* and good suppression of *Matricaria chamomilla*, *Stellaria media*, *Veronica persica* and *Viola* spp.

Pyraflufen-ethyl is also used as the defoliant for cotton and as a desiccant for potatoes. Pyraflufen-ethyl is a novel inhibitor of protoporphyrinogen IX oxidase.[2] Inhibition of this enzyme in chloroplasts causes accumulation of protoporphyrinogen IX, which results in peroxidation of foliar cell membrane lipids under the light and finally death of cells.

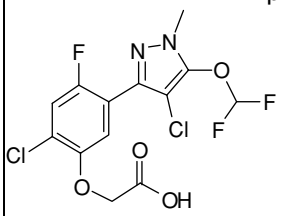
This herbicidal mode of action of pyraflufen-ethyl is similar to those of other peroxidizing herbicides containing a diphenyl ether moiety. Herbicidal effects of pyraflufen-ethyl are revealed as yellowing and browning in the foliar portion, and then death of the whole plant with leaf burn.

Selectivity between wheat and *Galium aparine* is based on differences in deposition, absorption and metabolism in both plants.

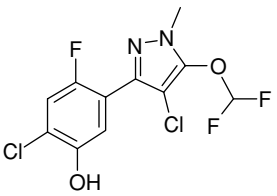
Regulatory position

The major metabolite of pyraflufen-ethyl in plants and soils is E-1 (ester hydrolysate). E-2 (phenol derivative) and E-3 (methylated E-2) are also detected as major metabolites in soils. The target analytes are considered to be pyraflufen-ethyl at least in plant materials, pyraflufen-ethyl, E-1, E-2 and E-3 in soils and pyraflufen-ethyl and E-1 in water samples.

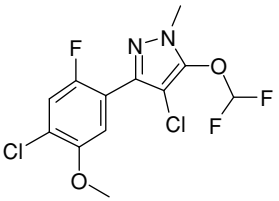
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E-1



E-2



E-3

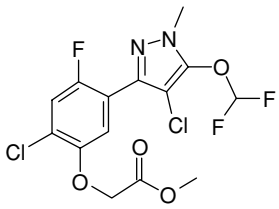
2 Outline of method

Since the target analytes depend entirely on the regulations in each country and on the sample matrices, residue analytical methods applicable to all matrices and analytes

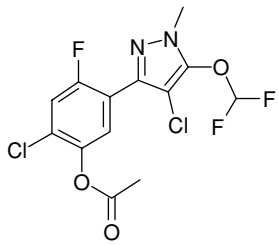
(pyraflufen-ethyl, E-1, E-2 and E-3) are provided. Because of the low application rate of pyraflufen-ethyl, highly sensitive residue analytical methods are required for pyraflufen-ethyl, E-1, E-2 and E-3. For this purpose, two different methods have been developed, a *'Multi-residue analytical method'* and a *'Total toxic residue analytical method'*.

A The *'Multi-residue analytical method'* is provided for plant, soil and water samples.

Sample extracts are cleaned up with a cartridge column before the acetylation of E-2 to E-16 and of E-1 to E-15. The final cleanup, plant material and soil samples are analyzed by gas chromatography (GC)/MSD. The GC/NPD method is applicable to water samples.



E-15



E-16

B The *'Total toxic residue analytical method'* is provided for plant, soil and water samples.

All of the compounds (pyraflufen-ethyl and its metabolites) are converted to E-2 and quantified as the total toxic residue of pyraflufen-ethyl. The conversion to E-2 is carried out by oxidative decomposition with concentrated sulfuric acid. The reaction mixture is extracted with a solvent and subjected to simple cleanup, followed by GC/NPD analysis. This method is rapid and simple compared with the *'Multi-residue analytical method'*, and has wide applicability to different varieties of the samples, such as plant materials, soils and water, with only minor adjustment of the analytical method.

3 Multi-residue analytical method

3.1 Apparatus

Blender or mill
Laboratory mechanical shaker
Vacuum-filtration system (Kiriyama funnel or equivalent)
Filter paper, No. 4 for Kiriyama funnel or equivalent
Erlenmeyer flasks, 100-, 200- and 500-mL
Round-bottom flasks, 200- and 1000-mL
Rotary vacuum evaporator
Block heater, with N_2 gas evaporation head
Separatory funnel, 150-mL
Eppendorf tubes, 1.5- and 2-mL

Fused-silica capillary column, HP-5MS, 30 m × 0.25-mm i.d., 0.25-μm film thickness, (5% phenyl)-methylpolysiloxane
Fused-silica capillary column, DB17, 15 m × 0.25-mm i.d., 0.15-μm film thickness, (50% phenyl)-methylpolysiloxane
Hewlett-Packard Model 6890 gas chromatograph with capillary split/splitless inlet with HP5973 mass-selective detector equipped with an autosampler
Shimadzu GC17A gas chromatograph with capillary split/splitless inlet with flame thermionic detector equipped with an AOC-17 autoinjector

3.2 Reagents and supplies

Acetonitrile, reagent grade and HPLC grade
Distilled water (prepared with a Yamato WG-75 system or HPLC grade)
Concentrated hydrochloric acid, reagent grade
Sodium chloride, reagent grade
Sodium sulfate, anhydrous
Ethyl acetate, reagent grade
n-Hexane, reagent grade
Toluene, reagent grade
Acetone, reagent grade
Triethylamine, reagent grade
Acetic anhydride, reagent grade
Methanol, reagent grade
Trimethylsilyldiazomethane, 10% in hexane solution
Bond Elut LRC C_{18} (500-mg), Varian
Bond Elut SAX (100-mg), Varian
Bond Elut SI (100-mg), Varian
Air, synthetic
Nitrogen, repurified
Helium, repurified
Hydrogen, synthetic

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3.3 Procedure

3.3.1 Extraction

(1) For plant materials

Weigh 20 g of the plant sample into an Erlenmeyer flask and add 40 mL of 1 N HCl and 160 mL of acetonitrile. Shake the flask for 30 min at 300 strokes min^{-1} using a shaker. Filter the aqueous acetonitrile extract through a No. 4 Kiriyama funnel filter paper. Wash the residue on the filter with 100 mL of acetonitrile. Combine the filtrates and remove acetonitrile with a rotary evaporator. Transfer the residue with 20 mL of saturated aqueous sodium chloride solution into a separatory funnel, extract the solution with 3 × 30 mL of n-hexane–ethyl acetate (9 : 1, v/v), and collect the organic phase in a flask. Dry with anhydrous sodium sulfate and remove the combined organic phase with a rotary evaporator. Transfer the residue into the Eppendorf tube with a

small amount of acetone and concentrate under an N_2 gas flow at about 40 °C. Re-suspend the remaining residue with 1 mL of acetone.

(2) Soil

The air-dried soils (50 g) are processed similarly to the plant materials.

(3) Water

Adjust to pH 2 by addition of concentrated HCl to 500 mL of water sample. Apply this acidic water to the Bond Elut LRC C_{18} (500-mg) cartridge column. Pass the elution solvent (2 mL of acetonitrile) through the cartridge and collect in a test-tube. Concentrate the eluate under an N_2 gas flow at about 40 °C.

3.3.2 Cleanup

(1) First cleanup

Pass 0.5 mL of the suspended acetone solution through the Bond Elut SCX (100-mg) cartridge and collect in an Eppendorf tube. Wash the cartridge column with two portions of 0.5 mL of acetone and combine all of the eluates (total volume: ca 1.5 mL). This step is omitted for water.

(2) Acetylation of E-2

Add 150 μL of the acetylating solution [triethylamine–acetic anhydride (2 : 1, v/v)] into the Eppendorf tube and allow to stand for about 30 min at room temperature. Concentrate the reaction mixture under an N_2 gas flow at about 40 °C. Add 1 mL of 0.1 N HCl to the residue and extract the solution with 3 × 0.5 mL of n-hexane–ethyl acetate (4 : 1, v/v). Concentrate the organic phase under an N_2 gas flow at about 40 °C.

(3) Methylation of E-1

Suspend the residue with 1 mL of acetone. Add 50 μL of methanol and 100 μL of 10% trimethylsilyldiazomethane–hexane solution and allow to stand for about 30 min at room temperature. Concentrate the reaction mixture under an N_2 gas flow at about 40 °C.

(4) Second cleanup

Add 2 × 0.5 mL of n-hexane–ethyl acetate (9 : 1, v/v) to the residue and apply to the Bond Elut SI (100-mg) cartridge column. Pass the elution solvent [1 mL of n-hexane–ethyl acetate (3 : 2, v/v)] through the cartridge and collect in an Eppendorf tube. Concentrate the eluate under an N_2 gas flow at about 40 °C and dissolve the residue in 0.5 mL of acetone. Dilute an aliquot of the acetone solution twofold with acetone and adjust the amount of impurities in both the standard and sample solutions for high reliability of GC analyses [details are shown in Section 3.3.4(1)].

3.3.3 Determination

Inject an aliquot of the GC-ready sample solution into the GC/MSD or GC/FTD system.

Operating conditions for GC/MSD

Gas chromatograph	Model GC6890, Hewlett-Packard
Sample injector	Pulsed splitless sample injector. Column head pressure, 98 kPa; pulse pressure, 240 kPa; pulse time, 0.5 min
Column	Fused-silica capillary column, HP-5MS, 30 m × 0.25-mm i.d., 0.25-μm film thickness
Column temperature	Initial 120 °C, held for 2 min, increased at 40 °C min^{-1} to 300 °C, held for 1 min
Injection port temperature	230 °C
Interface temperature	250 °C
Detector	Mass-selective detector, MSD5973, electron ionization energy 70 eV, ion source temperature 135 °C
Selected ion monitoring	m/z 412 (pyraflufen-ethyl), 398 (E-15), 326 (E-16) and 340 (E-3)
Gas flow rate	Helium carrier gas, 1.2 mL min^{-1}
Injection volume	1 μL

Operating conditions for GC/NPD

Gas chromatograph	Model GC17A, Shimadzu
Sample injector	Splitless sample injector. Column head pressure: 100 kPa
Column	Fused-silica capillary column, DB-17, 15 m × 0.25-mm i.d., 0.15-μm film thickness
Column temperature	Initial 80 °C, held for 1 min, increased at 50 °C min^{-1} to 200 °C, held for 2–3.5 min, then increased at 2 °C min^{-1} to 210 °C and at 2.5 °C min^{-1} to 223 °C
Injection port temperature	250 °C
Detector	Flame thermionic detector
Detector temperature	280 °C
Gas flow rates	Helium carrier gas, 2.6 mL min^{-1} Hydrogen, 50 kPa Air, 70 kPa
Injection volume	1 μL

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3.3.4 Evaluation

(1) Method

(a) Standardization

For GC/MSD: Peaks of the analytes usually appear at retention times around 6.9, 6.8, 6.3 and 6.0 min for pyraflufen-ethyl, E-15 (E-1), E-16 (E-2) and E-3, respectively.

Construct a calibration curve by plotting the peak area against the standard concentration to obtain a least-squares regression line.

For GC/NPD: Peaks of the analytes usually appear at retention times around 11.8, 11.3, 7.2 and 6.5 min for pyraflufen-ethyl, E-15 (E-1), E-16 (E-2) and E-3, respectively. Plot the peak areas against the concentrations of the analytes.

Recommendation: Dilute the standard solutions twice with blank solutions prepared from each of the blank samples. Impurities in the blank samples reduce the thermal decomposition of the target analytes in the injection port and stabilize the profiles of ionization and fragmentation of the target analytes.

(b) Detection of sample residues

Inject the cleaned-up sample into the GC/MSD or GC/FTD system operated under the same conditions as employed for standardization. Compare the peak areas of the analytical samples with the calibration curve. Determine the concentrations of pyraflufen-ethyl, E-15 (E-1), E-16 (E-2) and E-3 present in the sample.

(2) Recoveries, limit of detection and limit of determination

With fortification levels between 0.002 and 0.03 mg kg^{-1}, recoveries from blank soils ranged from 72 to 103% with the limit of quantitation being 0.002 mg kg^{-1}. With fortification levels between 0.001 and 0.005 mg kg^{-1}, recoveries from blank water ranged from 82 to 103% with the limit of quantitation being 0.001 mg kg^{-1}.

(3) Calculation of residues

Calculate the concentrations of analytes (pyraflufen-ethyl, E-1, E-2 and E-3) in plant, soil and water samples (mg kg^{-1} or $\mu g\,L^{-1}$) using the following equation:

$$\text{Analyte concentration} = \frac{A \times V \times F}{W}$$

where

A = concentration of the analyte in the final solution (mg kg^{-1} or $\mu g\,L^{-1}$)
V = volume of the final solution (mL)
W = weight or volume of analysis sample (g or mL)
F = molecular weight factor: pyraflufen-ethyl = 1, E-1/E-15 = 0.965, E-2/E-16 = 0.886, E-3 = 1

3.3.5 Important points

Pyraflufen-ethyl and E-15 (methyl ester of E-1) are easily hydrolyzed to E-1. Careful handling is recommended to prevent the hydrolysis of these analytes.

4 Total toxic residue analytical method

4.1 Apparatus

Blender or mill
Laboratory mechanical shaker
Vacuum-filtration system (Kiriyama funnel or equivalent)
Filter paper, No. 4 for Kiriyama funnel or equivalent
Erlenmeyer flasks, 100-, 200- and 500-mL
Round-bottom flasks, 200- and 1000-mL
Separatory funnel, 150-mL
Rotary vacuum evaporator
Block heater with N_2 gas evaporation head
Eppendorf tubes, 1.5- and 2-mL
Fused-silica megabore column, DB17, 15 m × 0.53-mm i.d., 1-μm film thickness, (50% phenyl)-methylpolysiloxane
Hewlett-Packard Model 5890 gas chromatograph with capillary split/splitless inlet with nitrogen–phosphorus detector equipped with a Model 7673A autosampler

4.2 Reagents and supplies

Acetonitrile, reagent grade and HPLC grade
Distilled water (prepared with a Yamato WG-75 system or HPLC grade)
Concentrated hydrochloric acid, reagent grade
Concentrated sulfuric acid, reagent grade
Sodium chloride, reagent grade
Sodium sulfate, anhydrous
Ethyl acetate, reagent grade
n-Hexane, reagent grade
Toluene, reagent grade
Acetone, reagent grade
Methanol, reagent grade
Trimethylsilyldiazomethane, 10% in hexane solution
Bond Elut LRC C_{18} (500-mg), Varian
Bond Elut SAX (100-mg), Varian
Bond Elut SI (100-mg), Varian
Air, synthetic
Nitrogen, repurified
Helium, repurified
Hydrogen, synthetic

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4.3 Procedure

4.3.1 Extraction

The sample extraction procedures are similar to those for the *'Multi-residue analysis method'* (see Section 3.3.1).

4.3.2 Cleanup

(1) First cleanup

Pass 0.5 mL of the suspended acetone solution through the Bond Elut SCX (100-mg) cartridge and collect in a test-tube. Wash the cartridge column with 2×0.5 mL of acetone and combine all of the eluates (total volume: ca 1.5 mL). Cleanup is not required for water samples.

(2) Methylation of E-1

Suspend the residue with 1 mL of acetone. Add 50 μL of methanol and 100 μL of 10% trimethylsilyldiazomethane–hexane solution and allow to stand for about 30 min at room temperature. Concentrate the reaction mixture under an N_2 gas flow at about 40 °C. This step is necessary for good accuracy of the quantitation of E-1 (details are given in Section 8B).

(3) Sulfuric acid reaction

Add 1 mL of concentrated sulfuric acid to the residue and keep at 100 °C for 3 h. Stop the reaction by addition of 5 mL of distilled water and follow by extraction with 3×2 mL of n-hexane–ethyl acetate (4 : 1, v/v). Concentrate the organic phase under an N_2 gas flow at about 40 °C.

(4) Second cleanup

Suspend the residue with 0.25 mL of n-hexane–ethyl acetate (9 : 1, v/v) and apply to the Bond Elut SI (100-mg) cartridge column. Wash the test-tube with 0.25 mL of n-hexane–ethyl acetate (9 : 1, v/v) and with 0.75 mL of n-hexane–ethyl acetate (4 : 1, v/v) and apply to the cartridge column. Pass the elution solvent [0.5 mL of n-hexane–ethyl acetate (3 : 1, v/v) and 0.5 mL of n-hexane–ethyl acetate (2 : 1, v/v)] through the cartridge and collect into an Eppendorf tube (total volume: ca 1 mL). Concentrate the eluate under an N_2 gas flow at about 40 °C and dissolve the residue in 1 mL of toluene.

4.3.3 Determination

Inject an aliquot of the GC-ready sample solution into GC/NPD system.

Operating conditions

Gas chromatograph	Model GC5890, Hewlett-Packard
Sample injector	Splitless sample injector
Column	Fused-silica megabore column, DB-17, 15 m × 0.53-mm i.d., 1-μm film thickness
Column temperature	200 °C
Injection port temperature	250 °C
Detector	Nitrogen–phosphorus detector

Detector temperature	250 °C
Gas flow rates	Helium carrier gas, 13 mL min^{-1}
	Hydrogen, 4 mL min^{-1}
	Air, 110 mL min^{-1}
Injection volume	2 μL

4.4 Evaluation

4.4.1 Method

(1) Standardization

The peak of E-2 usually appears at a retention time around 3 min. Plot the peak area against the concentration of E-2.

(2) Detection of sample residues

Inject the cleaned-up sample into the GC/NPD system operated under the same conditions as used for standardization.

Compare the peak areas of the analytical samples with the calibration curve. Determine the concentration of E-2 present in the sample using following equation:

$$\text{E-2 concentration} = \frac{A \times V}{W}$$

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where

A = concentration of E-2 in the final solution (mg kg^{-1} or mg L^{-1})
V = volume of the final solution (mL)
W = weight or volume of analysis sample (g or mL)

4.4.2 Recoveries, limit of detection and limit of determination

With fortification levels of 0.20 mg kg^{-1}, recoveries from untreated plant matrices ranged from 90 to 102%. The limit of quantitation is 0.006 mg kg^{-1}.

4.4.3 Calculation of residues

Calculate the total residue in terms of total pyraflufen-ethyl in plant, soil and water samples (mg kg^{-1} or mg L^{-1}) using the following equation:

$$\text{Total pyraflufen-ethyl concentration} = B \times F$$

where

B = concentration of E-2 in the final solution (mg kg^{-1} or mg L^{-1})
F = molecular weight factor for pyraflufen-ethyl/E-2 = 1.263

4.5 Important points

Pyraflufen-ethyl and E-15 (methyl ester of E-1) are easily hydrolyzed to E-1. Careful handling is recommended to prevent the hydrolysis of these analytes.

Pyraflufen-ethyl (ester type) and E-3 are quantitatively converted to E-2 by the sulfuric acid treatment, but not E-1 (free acid type); therefore, methylation of E-1 to E-15 (ester type) is a necessary step for accurate analysis of total residues.

References

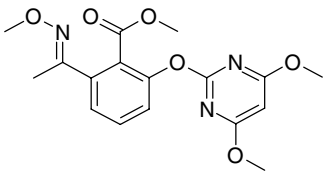
1. Y. Miura, M. Ohnishi, T. Mabuchi, and I. Yanai, 'ET-751: A new herbicide for use in cereals,' in "Proceedings of the 1993 Brighton Crop Protection Conference–Weeds," vol. 1, pp. 35–40 (1993).
2. S. Murata, A. Yuda, A. Nakano, Y. Kimura, K. Motoba, T. Mabuchi, Y. Miura, H. Nishizawa, and S. Funayama, 'Mechanisms of selective action of the peroxidizing herbicide ET-751 on wheat and *Galium aparine*,' in "Proceedings of the 1995 Brighton Crop Protection Conference–Weeds," vol. 1, pp. 243–248 (1995).

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Pyriminobac-methyl

Materials to be analyzed	Rice (grain, straw) and soil
Instrumentation	Gas-chromatographic determination

1 Introduction

Chemical name (IUPAC)	Methyl 2-(4,6-dimethoxy-2-pyrimidinyloxy)-6-(1-methoxyiminoethyl)benzoate	
Structural formula		
Empirical formula	$C_{17}H_{19}N_3O_6$	
Molar mass	361.4	
Isomer	*E*-isomer	*Z*-isomer
Melting point	107–109 °C	70 °C
Vapor pressure	0.035 mPa at 25 °C	0.02681 mPa at 25 °C
Solubility	Water 9.25 μg L^{-1} (20 °C)	0.175 g L^{-1} (20 °C)
	Methanol 14.6 g L^{-1} (20 °C)	14.0 g L^{-1} (20 °C)
Stability	Stable in water (>1 yr, pH 4–9) and to heat (no decomposition after 14 days at 55 °C)	
Use pattern	Pyriminobac-methyl is a selective, systemic, early post-emergence herbicide to control barnyard grass in paddy rice fields	
Regulatory position	The residue definition for pyriminobac-methyl is for the combined *E*- and *Z*-isomers.	

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2 Outline of method

Pyriminobac-methyl in plant samples (rice grains and rice straw) and soil is recovered by refluxing with aqueous acetone. After removing acetone from the extract, pyriminobac-methyl in the aqueous solution is transferred into n-hexane. The n-hexane layer is dried and evaporated under reduced pressure. The residue from soil

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samples is purified with an alumina column. The residue from plant samples is further cleaned up by cartridge column chromatography. Pyriminobac-methy is determined by gas chromatography (GC) with nitrogen–phosphorus detection (NPD). Residue levels are reported as the sum of both the *E*- and *Z*-isomers.

3 Apparatus

Mill (coffee-mill type)
Grinder (Cutting Mills, Willey type)
Round-bottom flasks, 500- and 300-mL
Pear-shaped flasks, 300- and 50-mL
Separatory funnel, 200-mL
Glass funnel 10-cm i.d.
Bell jar-type filtering apparatus
Buchner funnel, 11-cm i.d.
Rotary vacuum evaporator, 40 °C bath temperature
Water-bath, electrically heated, temperature 80 °C
Condenser
Glass chromatography column, 1.5-cm i.d. × 45 cm with a stopcock
Alumina column: Place a cotton wool plug at the bottom of a glass chromatography column. Pack 15 g of alumina slurried with n-hexane–ethyl acetate (6 : 1, v/v) into the glass column. Make an anhydrous sodium sulfate layer of about 1 cm above and below the alumina bed
Gas chromatograph, equipped with a nitrogen–phosphorus detector
Microsyringe, 10-μL

4 Reagents

Acetone, n-hexane, acetonitrile, ethyl acetate, pesticide residue analysis grade
Aluminum oxide, Aluminumoxid 90, activity II–III, 70–230 mesh MSTM (Merck)
Anhydrous sodium sulfate, sodium chloride, special grade
Distilled water, HPLC grade
Cartridge chromatography column: Sep-Pak Plus NH_2 cartridges and Sep-Pak Plus Silica cartridges (Waters)
Filter aid, Celite 545 (Johns-Manville Products Corporation)
Filter paper, 11-cm i.d.
Pyriminobac-methyl, *E*-isomer, *Z*-isomer, analytical grade (Ihara Chemical Industries Co., Ltd)
Pyriminobac-methyl standard solutions: 0.05, 0.15, 0.25, 0.5, 1.0 and 1.5 μg mL^{-1} in acetone (*E*- and *Z*-isomer, separately)

5 Sampling and sample preparation

Collect 1 kg of rice grains and grind with a mill. Collect about 1 kg of rice straw and grind with a grinder. Soil, collected from the top 10-cm surface layer, is homogenized and passed through a 5-mm sieve.

6 Procedure

6.1 Extraction

6.1.1 Rice grains

Weigh 20 g of the sample into a 300-mL round-bottom flask and add 20 mL of distilled water to swell the sample for 1 h. Add 80 mL of acetone and reflux the sample at 80 °C for 1 h after attaching a condenser. Filter the extract through a filter paper on a Buchner funnel with suction into a 500-mL round-bottom flask. Wash the flask and the residue with 100 mL of acetone and filter the washings in a similar manner. Combine the filtrates and concentrate to approximately 20 mL under reduced pressure at 40 °C.

6.1.2 Rice straw

Weigh 5 g of the sample into a 500-mL round-bottom flask and add 50 mL of distilled water to swell the sample for 2 h. Add 150 mL of acetone and reflux the sample at 80 °C for 1 h after attaching a condenser. Conduct the subsequent procedures in a similar manner as for rice grains and concentrate the filtrates to approximately 50 mL.

6.1.3 Soil

Weigh 40 g (dry soil weight) of the sample into a 500-mL round-bottom flask and add 200 mL of acetone–water (3 : 1, v/v). Reflux the sample at 80 °C for 1 h after attaching a condenser. After cooling the extract, add 5 g of Cellite to the flask, mix the contents well and filter the extract. Conduct the subsequent procedures in a similar manner as for rice grains and concentrate the filtrates to approximately 50 mL.

DropBooks

6.2 Liquid–liquid partition (rice grain, rice straw and soil)

Transfer the concentrate from Section 6.1 to a 200-mL separatory funnel with 50 mL of water and add 10 mL of saturated aqueous sodium chloride solution. Extract twice with 100 mL of n-hexane. Dry the n-hexane extract by passing through about 80 g of anhydrous sodium sulfate on a glass funnel into a 500-mL separatory funnel for the rice samples and into a 500-mL round-bottom flask for the soil sample. Wash the anhydrous sodium sulfate with 30 mL of n-hexane and combine the washings into the vessel. The n-hexane extract of soil sample is evaporated to dryness under reduced pressure, then the soil residue is processed as described in Section 6.3.2.

6.3 Cleanup

6.3.1 n-Hexane–acetonitrile partition (rice grain and rice straw)

Extract the n-hexane layer in the separatory funnel (from Section 6.2) twice with 100 mL of acetonitrile. Collect the acetonitrile extract in a 300-mL round-bottom flask and evaporate the acetonitrile under reduced pressure.

6.3.2 Alumina column chromatography (rice grain, rice straw and soil)

Dissolve the residue in 3 mL of n-hexane–ethyl acetate (6 : 1, v/v) and adsorb on the top of an alumina column bed. Rinse the flask three times with 1 mL of the same solvent mixture and transfer the rinsings to the column. Elute interfering substances with 50 mL of the same solvent mixture and discard the eluate. Then elute pyriminobac-methyl with 150 mL of the same solvent mixture. Collect the eluate in a 300-mL pear-shaped flask and evaporate to dryness under reduced pressure. The residue of the soil sample is dissolved in an appropriate volume of acetone for GC analysis.

6.3.3 Cartridge column chromatography (rice grain and rice straw)

Connect a Sep-Pak Plus NH_2 column with a Sep-Pak Plus Silica column (place the silica column on the elution side). Condition the connected column with 5 mL of n-hexane. Dissolve the residue of rice grains and rice straw (Section 6.3.2) in a small volume of n-hexane and transfer the solution to the column. Elute with 6 mL of n-hexane–ethyl acetate (9 : 1, v/v) and discard the eluate. Remove the NH_2 column. Elute the Sep-Pak Plus Silica column with 15 mL of n-hexane–ethyl acetate (4 : 1, v/v) and collect the eluate in a 50-mL pear-shaped flask.

Evaporate the solvent to dryness under reduced pressure and dissolve the residue in an appropriate volume of acetone for GC analysis.

6.4 Gas-chromatographic determination

Inject an aliquot (V_i) of the solution prepared from Section 6.3.2 (V_{End}) for soil and Section 6.3.3 (V_{End}) for rice (grain, straw) into the gas chromatograph.

Operating conditions

Gas chromatograph	Hewlett-Packard Model 5890
Column	DB-1301, 30 m × 0.53-mm i.d., film thickness 1.0-μm
Column temperature	220 °C
Injection port temperature	230 °C
Detector	Nitrogen–phosphorus detector
Detector temperature	240 °C
Gas flow rates	Helium carrier gas, 20 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 100 mL min^{-1} Helium make-up gas, 10 mL min^{-1}
Integrator	Hewlett-Packard Model 3396A
Attenuation	2
Chart speed	10 mm min^{-1}
Injection volume	1–4 μL
Retention time	*E*-isomer, 6.06 min, *Z*-isomer, 4.43 min
Minimum detectable amount	*E*-isomer, 0.2 ng, *Z*-isomer, 0.2 ng

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Construct a fresh calibration curve with pyriminobac-methyl standard solutions (individual isomer) for each set of analyses.

Inject 4 μL of each pyriminobac-methyl standard solution into the gas chromatograph. Using log–log paper, plot the peak heights in millimeters against the injected amount of pyriminobac-methyl in nanograms. For the heights of the peaks obtained for these solutions, record the appropriate amount of pyriminobac-methyl from the calibration curve. The residue level is calculated as the sum of both the *E*- and *Z*-isomers.

7.2 Recoveries and limit of detection

The recoveries from control samples fortified with pyriminobac-methyl (for individual isomers) at levels of 0.1 mg kg^{-1} were 96–106% for rice grains, 80–90% for rice straw and 77–92% for soil. The limit of detection was 0.005 mg kg^{-1} for rice grains, 0.01 mg kg^{-1} for rice straw and 0.005 mg kg^{-1} for soil.

7.3 Calculation of residues

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The residue R, expressed in mg kg^{-1} pyriminobac-methyl (for individual isomers), is calculated using the following equation:

$$R = (W_A \times V_{End})/(V_i \times G)$$

where

G = sample weight (g)
V_{End} = terminal volume of sample solution from Section 6.3 (mL)
V_i = portion of volume V_{End} injected into the gas chromatograph (μL)
W_A = amount of pyriminobac-methyl for V_i read from calibration curve (ng)

8 Important points

8.1 Method for extraction of pyriminobac-methyl from soil

The method was developed/validated using two typical rice field soils (alluvial soil and volcanic ash soil). In the extraction efficiency study, a soil sample that had been incubated for about 8 months under flooded conditions after addition of

pyriminobac-methyl (*E*-isomer) at a level of 5 mg kg^{-1} was used. In the reflux extraction, there was no observable difference in the extraction efficiency between acetone, acetonitrile and methanol for alluvial soil (0.112, 0.113 and 0.107 mg kg^{-1}, respectively). For volcanic ash soil, however, there was some observable difference. The extraction efficiency was higher with acetone and slightly lower with acetonitrile, and that with methanol was about half of that with acetone (acetone 0.438, acetonitrile 0.378 and methanol 0.211 mg kg^{-1}).

Extraction efficiencies with shaking and ultrasonic extraction were lower than those with solvent reflux extraction.

In alluvial soil, there was no great difference in the extraction efficiencies among the various solvent combinations tested, with acetone, acidic acetone or alkaline acetone, for reflux extraction.

In the soil metabolism study using [^{14}C]pyriminobac-methyl, most of the residual pyriminobac-methyl in soil was extracted by reflux extraction with acetone.[1]

8.2 Extraction of pyriminobac-methyl from rice grain and rice straw

Reflux extraction with acetone has been used for the analysis of rice grain and rice straw including metabolites, and showed good recoveries.[2]

8.3 Cleanup

For soil samples, sufficient sample cleanup could be conducted even if the alumina column was changed to a Sep-Pak Alumina N cartridge (Waters) by the following process. The entire sample of the dried n-hexane extract (Section 6.2) is introduced into a Sep-Pak Alumina N cartridge, and the column is washed with 50 mL of n-hexane. Subsequently, pyriminobac-methyl is eluted with 3 mL of ethyl acetate, the solvent is evaporate to dryness under reduced pressure and the residue is dissolved in an appropriate volume of acetone for GC analysis.

8.4 GC column

If interfering peaks hinder sample quantitation on the gas chromatogram, better resolution could be obtained by using an FFAP or DB-17 gas chromatographic column.

8.5 Sample storage stability

Pyriminobac-methyl (*E*- and *Z*-isomer, separately) in rice grain (0.1 mg kg^{-1}), rice straw (0.1 mg kg^{-1}) and soil (0.02 mg kg^{-1}) are stable for up to 60 days when stored at −20 °C.

References

1. Y. Nagai, K. Shinba, A. Yagi, and Y. Yusa, 'Degradation of a new herbicide, pyriminobac-methyl, in soils,' in "Abstracts of the 21st Annual Meeting of the Pesticide Science Society of Japan," p. 35 (1996).
2. Y. Saito, H. Matsushita, Y. Nagai, Y. Asano, and Y. Yusa, 'Residue analysis of pyriminobac-methyl and its metabolites in rice and soil,' in "Abstracts of the 21st Annual Meeting of the Pesticide Science Society of Japan," p. 138 (1996).

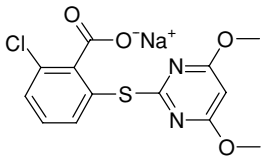
Akira Yagi, Mitsumasa Ikeda and Yoshihiro Saito
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DropBooks

Pyrithiobac-sodium

Materials to be analyzed	Cottonseeds and soil
Instrumentation	Gas-chromatographic determination

1 Introduction

Chemical name (IUPAC)	Sodium 2-chloro-6-(4,6-dimethoxypyrimidin-2-ylthio)-benzoate
Structural formula	
CAS No.	123343-16-8
Empirical formula	$C_{13}H_{10}ClN_2NaO_4S$
Molar mass	348.7
Melting point	233.8–234.2 °C (decomp.)
Vapor pressure	4.8×10^{-6} mPa (25 °C)
Solubility	Water 72.8 g L^{-1} (20 °C)
Use pattern	Pyrithiobac-sodium is a pre- and post-emergence herbicide to control broad-leaved weed and grasses
Regulatory position	The residue definition is for the parent, pyrithiobac-sodium, only.

2 Outline of method

Pyrithiobac-sodium is extracted as the free acid, pyrithiobac, from crop and soil samples.

For cottonseeds, pyrithiobac-sodium is extracted with acetone–water (4 : 1, v/v). After filtration, the acetone is removed by evaporation under reduced pressure. The residue is adjusted to pH 1 and extracted with ethyl acetate. The extract is cleaned up by liquid–liquid partitioning and methylated with diazomethane. The methyl ester of pyrithiobac is purified by silica gel column chromatography. Pyrithiobac-methyl is determined by gas chromatography (GC) with nitrogen–phosphorus detection (NPD).

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For soil, acetone–1 N sulfuric acid (4 : 1, v/v) is added to the soil sample and the mixture is refluxed. The subsequent procedures are as for cottonseeds.

3 Apparatus

Mill (coffee-mill type)
Buchner funnel, 11-cm i.d.
Funnel, 10-cm diameter
Erlenmeyer flasks, 500- and 1000-mL
Round-bottom flasks, 1000-, 500- and 300-mL with ground joints
Reflux condenser
Rotary vacuum evaporator, 40 °C bath temperature
Separatory funnel, 300-mL
Glass chromatography column, 15-mm i.d. × 400 mm with a stopcock
Silica gel column: Place a cotton wool plug at the bottom of glass chromatography column and then add anhydrous sodium sulfate in a layer 1-cm thick. Weigh 10 g of silica gel and pour it into the tube with n-hexane–benzene (1 : 3, v/v). Rinse the silica gel column with the same solvent system and place an anhydrous sodium sulfate in a layer 1-cm thick on the top the column.
Gas chromatograph, equipped with a nitrogen–phosphorus detector
Microsyringe, 10-μL
Volumetric flasks, 5- and 10-mL
Filter paper, 110-mm diameter
pH test paper

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4 Reagents

Distilled water, high-performance liquid chromatography grade
Acetone, ethyl acetate, diethyl ether, acetonitrile, n-hexane, benzene, pesticide residue analysis grade
Pyrithiobac-sodium, analytical grade (KI Research Inst. Co. Ltd)
Pyrithiobac-methyl, analytical grade (KI Research Inst. Co. Ltd)
Sulfuric acid, hydrochloric acid, potassium hydroxide, *N*-methyl-*N*-nitroso-*p*-toluenesulfamide, anhydrous sodium sulfate, sodium chloride, special grade
Filter aid, Celite 545 (Johns-Manville Products Corporation)
Silica gel, Wako gel C-200, residual agricultural chemical grade (Wako Pure Chemical Industries, Ltd)
Diazomethane: Follow essentially Aldrich Chemical Company's procedure for the 'Preparation of ethereal–alcoholic solution of diazomethane': A 25-mL volume of ethanol is added to a solution of 5 g of potassium hydroxide in 8 mL of water in a 100-mL distillation flask fitted with a dropping funnel and a distillation condenser. The lower end of the condenser extends through and just below the neck of a 250-mL Erlenmeyer receiving flask, the latter being cooled in an ice-bath. The distillation flask containing the alkaline solution is heated in a water-bath to 65 °C and the contents of the flask are agitated with a magnetic stirrer. A solution of

21.5 g (0.1 mol) of *N*-methyl-*N*-nitroso-*p*-toluenesulfamide (Diazald) in about 130 mL of diethyl ether is added to the flask through a dropping funnel within about 25 min. The rate of distillation should be equal to the rate of addition. When the dropping funnel is empty, another 20 mL of diethyl ether are added slowly, and the distillation is continued until the distilling ether is colorless. The distillate should contain about 3 g of diazomethane. **Caution:** diazomethane is not only exceedingly toxic, but also its solutions have been known to explode unaccountably. Hence all work with diazomethane should be carried out in an efficient hood.

Pyrithiobac-methyl standard solutions: 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mg L^{-1} in acetone.

5 Sampling and sample preparation

The soil sample is prepared by manually removing stones and plant material and passing through a 5-mm sieve.

6 Procedure

6.1 Extraction

6.1.1 Cottonseeds

Weigh 10 g of the sample into a 500-mL Erlenmeyer flask. Add 120 mL of water–acetone (1 : 4, v/v) and shake the flask vigorously for 1 h. Filter the extract through a filter paper in a Buchner funnel into a 1-L round-bottom flask with suction. Rinse the flask and the filter cake twice with 50 mL of acetone. Combine the filtrates and concentrate to 20–30 mL under reduced pressure at 40 °C.

6.1.2 Soil

Weigh 30 g (dry soil) of the sample into a 500-mL round-bottom flask. Add 120 mL of 1 N sulfuric acid–acetone (1 : 4, v/v) and reflux the mixture at 80 °C for 3 h. Filter the extract through a filter paper overlaid with 20 g of Celite in a Buchner funnel into a 1-L round-bottom flask with suction. Rinse the flask and the filter cake twice with 50 mL of acetone and filter the acetone solution. Combine the filtrates and concentrate to 20–30 mL under reduced pressure.

6.2 Cleanup

6.2.1 Liquid–liquid partition

Transfer the residue prepared in Section 6.1.1 or 6.1.2 to a 300-mL separatory funnel with 50 mL of distilled water. Acidify the solution to pH 1 with 2.5 mL of 2 N hydrochloric acid and add 10 mL of saturated aqueous sodium chloride. Extract the aqueous mixture twice with 70 mL of ethyl acetate. Combine the extracts and filter into a 500-mL round-bottom flask through 60 g of anhydrous sodium sulfate supported by a plug of cotton wool in a funnel. Evaporate the filtrate to dryness under

reduced pressure. Dissolve the residue in 100 mL of n-hexane and extract twice with 80 mL of acetonitrile. Combine the acetonitrile layers and concentrate to approximately 0.5 mL under reduced pressure. Remove the last traces of solvent with a gentle stream of air. Dissolve the residue in 50 mL of distilled water and add 0.4 mL of 2 M potassium hydroxide and 10 mL of saturated aqueous sodium chloride. Extract the mixture twice with 70 mL of n-hexane and discard the n-hexane layers. Acidify the aqueous solution to pH 1 with 2.5 mL of 2 N hydrochloric acid and extract twice with 70 mL of ethyl acetate. Combine the extracts and filter through anhydrous sodium sulfate in a funnel into a 500-mL round-bottom flask. Evaporate the filtrate to dryness under reduced pressure.

For soil samples, omit the n-hexane–acetonitrile partition procedure.

6.2.2 Methylation

Add 6 mL of an ethereal solution of diazomethane (excess, ca 80 mg) to the residue prepared in Section 6.2.1. Allow the reaction mixture to stand at room temperature for 2 h. Evaporate the mixture to dryness under reduced pressure.

6.2.3 Column chromatography

Prepare a silica gel column as described in Section 3. Dissolve the residue prepared in Section 6.2.2 in 3 mL of n-hexane–benzene (1 : 3, v/v) and transfer the solution to the column. Rinse the flask twice with 5 mL of the same solvent system and also transfer these solutions to the column. Allow the solution to percolate through the column and discard the eluate. Add 190 mL of n-hexane–benzene (1 : 3, v/v) to the column. Discard the first 60 mL of eluate and collect the second 130 mL of eluate in a 300-mL round-bottom flask. Evaporate the eluate to dryness under reduced pressure.

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6.3 Gas-chromatographic determination

Dissolve the residue prepared in Section 6.2.3 in acetone. Transfer the solution to a volumetric flask, and make up to a given volume, e.g. 5 mL (V_{End}), with acetone. Inject an aliquot of the solution (V_i) into the gas chromatograph.

Operating conditions

Gas chromatograph	HP 5890A (Hewlett-Packard)
Column	DB-17, 1.0-μm thickness, 20 m × 0.53-mm i.d. (J&W Scientific)
Column temperature	230 °C
Injection temperature	250 °C
Detector	Nitrogen–phosphorus detector
Detector temperature	250 °C
Gas flow rates	Helium carrier gas, 20 mL min^{-1}
	Helium make-up, 10 mL min^{-1}
	Hydrogen, 3 mL min^{-1}
	Air, 100 mL min^{-1}

Attenuation	2^{-1}
Chart speed	0.5 cm min^{-1}
Injection volume	1–5 μL
Retention time for pyrithiobac-methyl	2.4 min

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Prepare a calibration curve by injecting pyrithiobac-methyl standard solutions, equivalent to 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 ng, into the gas chromatograph. Measure the heights of the peaks obtained. Plot the peak heights in millimeters against the injected amounts of pyrithiobac-methyl in nanograms.

Measure the peak heights of pyrithiobac-methyl on the chromatogram of the sample solution and quantify pyrithiobac-methyl by comparing the peak height with the calibration curve. Calculate the amount of pyrithiobac-sodium by multiplying the amount of pyrithiobac-methyl by a factor (1.023).

7.2 Recoveries, limit of detection and limit of determination

Untreated control samples of cottonseed and soil were fortified with pyrithiobac-sodium. The fortification levels were 0.01–0.1 mg kg^{-1} for cottonseeds and 0.005–0.05 mg kg^{-1} for soil. The recoveries obtained were 95–110% from cottonseeds and 86–98% from soil. The routine limit of determination was 0.01 mg kg^{-1} for cottonseeds and 0.005 mg kg^{-1} for soil.

7.3 Calculation of residues

The residue R, expressed in mg kg^{-1} pyrithiobac-sodium, is calculated using the following equation:

$$R = [(W_A \times V_{End})/(V_i \times G)] \times F$$

where

G = sample weight (g)
V_{End} = final volume of sample solution from Section 6.3 (mL)
V_i = portion of volume V_{End} injected into the gas chromatograph (μL)
W_A = amount of pyrithiobac-methyl for V_i read from the calibration curve (ng)
F = factor (1.023) for conversion from pyrithiobac-methyl (MW 340.8) to pyrithiobac-sodium (MW 348.7)

8 Important points

Alkylating reagents such as boron trifluoride–methanol, sulfuric acid–methanol, methanol–hydrochloric acid and methyl iodine–sodium hydride do not react efficiently with pyrithiobac.[1] Trimethylsilyldiazomethane may be used for the methylation of pyrithiobac.

Reference

1. H. Matsushita, T. Oishi, Y. Asano, and K. Ishikawa, 'Residue analytical method of pyrithiobac-sodium in crops and soil,' in Abstracts of the 17th Annual Meeting of the Pesticide Science Society of Japan, p. 149 (1992).

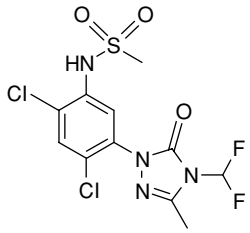
Yoshihiro Saito, Mitsumasa Ikeda and Akira Yagi
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DropBooks

Sulfentrazone

Materials to be analyzed	Soybean seed and processed parts (meal, hulls and oil); wheat grain, forage, hay; corn grain, forage, stover and processed parts (flour); rice grain, straw and processed parts (hulls, bran and polished rice); sorghum grain, forage and stover; tobacco (green, dried, cigarette, and smoke condensate); pea; alfalfa forage and hay; sunflower and processed parts (meal and oil); peanut nutmeat and processed parts (meal and oil); sugarcane and processed parts (refined sugar and molasses); and potato tuber and processed parts (wet peel, flakes and chips).
Instrumentation	Gas-chromatographic determination for plant matrices.

1 Introduction

Chemical name (IUPAC)	*N*-[2,4-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]phenyl]methanesulfonamide
Structural formula	
Empirical formula	$C_{10}H_{10}N_4O_3F_3SCl_2$
Molar mass	387.2
Melting point	121–123 °C
Physical state/odor	Light-tan powder with a musty odor
Vapor pressure	8×10^{-10} mmHg (25 °C)
Water solubility	400 mg L^{-1} (25 °C)
Specific gravity	1.21 g mL^{-1} (20 °C)
Stability	Stable at pH 5–9
Other properties	Undergoes photolysis in water rapidly. The compound is stable to photolysis in soil and is relatively persistent in soil, with a field half-life ($t_{1/2}$) of 121 days in sandy soil and $t_{1/2}$ of 302 days in clay soil.

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Use pattern

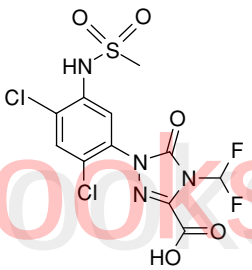
Sulfentrazone is a broad-spectrum, pre-emergent herbicide that provides good control over broadleaf weeds, grasses and sedges in crops and turf. The metabolism of sulfentrazone in animals and plants is similar. The major plant metabolite of sulfentrazone is 3-hydroxymethyl sulfentrazone (HMS). The soybean tolerance of 0.05 mg kg^{-1} includes residues of sulfentrazone plus its major metabolite, HMS. The rotational crop tolerance includes residues of sulfentrazone and its major metabolites, HMS and 3-desmethylsulfentrazone (DMS). The tolerance levels for cereal grains (excluding sweet corn) are as follows: 0.1 mg kg^{-1} in grain, 0.2 mg kg^{-1} in hay, 0.6 mg kg^{-1} in straw, 0.2 mg kg^{-1} in forage, 0.1 mg kg^{-1} in stover, 0.15 mg kg^{-1} in bran and 0.3 mg kg^{-1} in hulls.

Regulatory position

The residue of interest includes the parent sulfentrazone, HMS, sulfentrazonecarboxylic acid (SCA) and DMS.

Sulfentrazone-3-carboxylic acid (SCA)

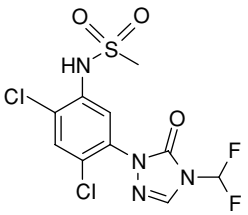
1-[2,4-dichloro-5-[*N*-(methylsulfonyl)amino]phenyl]-4-difluoromethyl-4,5-dihydro-5-oxo-1*H*-1,2,4-triazole-3-carboxylic acid



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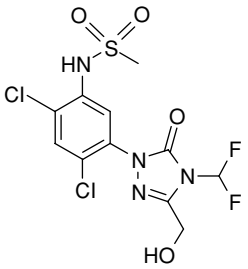
3-Desmethylsulfentrazone (DMS)

N-[2,4-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-5-oxo-1*H*-1,2,4-triazol-1-yl]phenyl]methanesulfonamide



3-Hydroxymethylsulfentrazone (HMS)

N-[2,4-dichloro-5-[4-(difluoromethlyl)-4,5-dihydro-3-hydroxymethyl-5-oxo-1*H*-1,2,4-triazol-1-yl]phenyl]methanesulfonamide



2 Method description

2.1 Method development history

The analytical method for sulfentrazone and its major plant metabolites originally included only a single hydrolysis step and analysis by gas chromatography with electron capture detection. During a radio-validation study (GC/ECD) analyzing plant samples from a metabolism study, it became apparent that the method did not account for all of the conjugated HMS and that the SCA was not completely converted to DMS. Consequently, the previous method was modified to include a more stringent hydrolysis step, to free all conjugated analytes of concern and to convert SCA completely to DMS. Additionally, a more specific detector was required to discriminate between the residues of sulfentrazone and its metabolites, and the matrix components released during the stringent hydrolysis step. A gas chromatograph equipped with an electrolytic conductivity detector (ELCD) or a halogen-specific detector (XSD) was utilized.

2.2 Outline of method

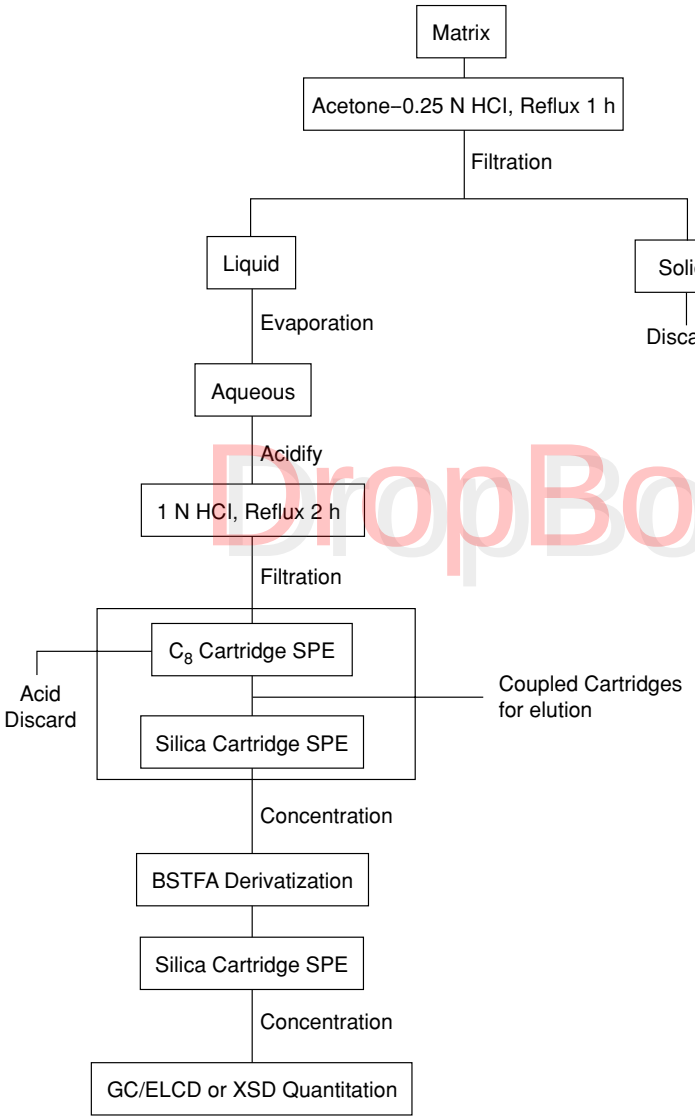
The enforcement method began with an acetone–0.25 N HCl reflux (1 h), filtration and concentration. The aqueous concentrate was acidified to 1 N, boiled under reflux (2 h), and filtered. The sample was then passed through a C_8 solid-phase extraction (SPE) cartridge and a silica gel SPE cartridge for clean-up. The eluate was concentrated and the HMS analyte was derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The derivatized solution was passed through a second silica gel SPE cartridge for additional cleanup. The eluate was concentrated and brought to a final volume with acetonitrile. Analysis was performed by gas chromatography (GC) with a 35% or 50% phenylmethylsilicone megabore or narrow-bore column.

This enforcement method has been validated on the raw agricultural commodity (RAC) and processed parts of various crops. For hay and straw matrices, the method limit of quantitation (LOQ) was validated at 0.05 mg kg^{-1} and the method limit of detection (LOD) was set at 0.01 mg kg^{-1}. For all other crop matrices, the LOQ was validated at 0.025 mg kg^{-1} and the LOD was set at 0.005 mg kg^{-1}. The method flow chart is presented in Figure 1.

3 Apparatus

AccessChrom or TurboChrom data acquisition software running on a MicroVax
Adapters, neoprene
Adapters, reducing
Balance, Analytical PM 2000, Mettler
Balance, top loading, Mettler
Boiling stones, Hengar
Buchner filter funnels, porcelain, 10.5-cm i.d., Coors
Capillary column, DB-5MS, 15 m × 0.25-mm i.d., 0.25-μm, J&W Scientific

Capillary column, DB-35MS, 15 m × 0.25-mm i.d., 0.25-μm, J&W Scientific
Capillary column, DB-17, 30 m × 0.54-mm i.d., 1-μm, J&W Scientific
Capillary column, DB-35, 30 m × 0.54-mm i.d., 1-μm, J&W Scientific
Centrifuge tubes, 13-mL, graduated, 0.1 mL, Pyrex
Condensers, Pyrex, Graham coil, 41 × 500 mm with ℸ 24/40 joint
Cylinders, graduated, 50-, 100-, 250-mL
Filter paper, Whatman No. 1, 11-cm diameter
Filter paper, Whatman GF/F (0.8 μm), 11-cm diameter



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Figure 1 Method flowchart for sulfentrazone determination.

Flasks, filter, 250-mL
Flasks, round-bottom boiling, 500-mL, T 24/40 joint
Flasks, volumetric, 100-mL
Gas chromatograph [Hewlett-Packard (HP) 5890 or 6890 GC with HP 7673 or 6890 Series injector and O I Analytical Model 5220 electrolytic conductivity detector or 5360 halogen-specific detector; HP 5890 or 6890 equipped with HP 7673 or 6890 Series injector and HP 5970 or 5972 mass-selective detector]
Heating mantles, Glas-Col
Injection port insert, cyclo-double gooseneck, Restek
Magnetic stirrers, VWR, Model 200
Microsyringes, Hamilton
Mill, Hobart
Mill, Wiley
Multi-tube vortexer, VWR
N-EVAP evaporator, Organomation
Pipets, disposable and volumetric
Reservoirs, plastic, 75-mL
Robot Coupe vertical cutter/mixer
SPE cartridge, silica gel (1-g), J.T. Baker
SPE cartridge, C_8 (1-g), Varian
Teflon stirring bars, VWR
TurboVap Evaporator, Zymark
TurboVap vessels, 200-mL, Zymark
TurboVap vessel support rack, Zymark
Visiprep manifold, Supelco
Visidry vacuum manifold drying attachment, Supelco

4 Reagents

Acetone, Resi-Analyzed, J.T. Baker
Acetonitrile, Resi-Analyzed, J.T. Baker
BSTFA, Pierce
Ethyl acetate, Resi-Analyzed, J.T. Baker
Hexane, Resi-Analyzed, J.T. Baker
Hydrochloric acid (HCl) (36.5–38.0%), J.T. Baker
Methanol, Resi-Analyzed, J.T. Baker
Equivalent equipment and reagents may be substituted as appropriate, unless specified otherwise in the method.

5 Sampling and preparation

Prior to analysis, samples should be chopped and finely pulverized with liquid nitrogen using a large Hobart (forage, hay, fodder and straw samples) or a Wiley mill (grain and seed samples). Recently, frozen crop matrices were processed more effectively with a Robot Coupe vertical cutter/mixer without liquid nitrogen.

6 Analytical procedures for nonoil crop matrices

6.1 Sample extraction, filtration and concentration

Weigh 10 g of the matrix into a 500-mL round-bottom boiling flask. For control samples to be fortified, add an accurately measured volume of a standard solution containing sulfentrazone, SCA and HMS uniformly to the matrix by syringe. Allow the solvent to evaporate (ca 1 min). Add 150 mL of acetone–0.25 N HCl (3 : 1, v/v) and a Teflon stirring bar or boiling stones. Place the round-bottom flask in a heating mantle and attach the flask to a cooling condenser. Gently boil the solution under reflux with stirring (if using a stirring bar) for 1 h.

Cool the sample extract to room temperature and filter the extract through a Whatman No. 1 (11-cm) filter paper (pre-rinsed with 5 mL of acetone) into a filter flask using a Buchner funnel and vacuum (15 inHg). Rinse the boiling flask with 2 × 25 mL of acetone and pass the rinsate through the post-reflux solid and filter paper. Transfer the filtrate into a 200-mL TurboVap vessel. Rinse the filter flask with 5 mL of acetone and add the rinsate to the TurboVap vessel. Concentrate the filtrate to <25 mL (not to dryness) using a TurboVap Evaporator (water-bath at 50 °C; increase the pressure up to 30 psi as the volume decreases). All traces of acetone must be removed.

6.2 Second reflux (conversion of SCA to DMS and release of conjugated HMS) *and filtration*

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Transfer the aqueous concentrate into a 500-mL round-bottom boiling flask. Rinse the TurboVap vessel with 2 × 5 mL of distilled water and add the rinsate to the round-bottom boiling flask. Add 3.5 mL of concentrated HCl to the aqueous concentrate to make the solution 1 N. Add a Teflon stirring bar or boiling stones. Place the round-bottom flask in a heating mantle and attach the flask to a cooling condenser. Gently boil the solution under reflux with stirring (if using a stirring bar) for 2 h.

Cool the sample extract to room temperature and filter the extract through a Whatman GF/F (11-cm) fine filter paper (pre-rinsed with 5 mL of distilled water) into a filter flask using a Buchner funnel and vacuum (15 inHg). Rinse the round-bottom boiling flask with 2 × 10 mL of distilled water and pass the rinsate through the post-reflux solid and filter paper. Transfer the filtrate into a 100-mL graduated mixing cylinder. Rinse the filter flask with 2 × 10 mL of distilled water, and add the rinsate to the mixing cylinder. Bring the volume up to 100 mL with distilled water. Shake the sample and take a 5-g (50-mL) aliquot.

6.3 C_8 SPE cartridge

Place a C_8 cartridge (1-g, Varian) on a vacuum manifold and condition the column with 6 mL of methanol followed by 6 mL of 0.25 N HCl. When conditioning SPE cartridges, allow the first conditioning solvent to reach the top of the cartridge packing before adding the second solvent. Maintain the flow rate through the C_8 cartridge at

5 mL min^{-1} by regulating the vacuum pump (5 inHg). The flow rate is more important than the vacuum pressure. Close the cartridge and add an additional 3 mL of 0.25 N HCl to the cartridge barrel. Attach a 75-mL plastic reservoir with an adapter to the top of the C_8 cartridge. Transfer the 50-mL aqueous sample aliquot to the reservoir. Pass the sample through the C_8 cartridge. Once the entire sample has passed through the C_8 SPE cartridge, use full vacuum briefly (2 min). Blow the cartridge completely dry with nitrogen using a manifold drying attachment (30 psi for at least 30 min). Return the C_8 SPE cartridge to the manifold and wash the cartridge with 6 mL of hexane–ethyl acetate (19 : 1, v/v). Remove the C_8 cartridge and prepare the first silica gel cartridge.

6.4 C_8 SPE cartridge/first slica gel SPE cartridge

Place a silica gel cartridge (1-g, J.T. Baker) on the vacuum manifold and condition with 6 mL of ethyl acetate followed by 6 mL of hexane. Do not allow the silica gel cartridge to go dry at any time during this step. Maintain the flow rate through the silica gel cartridge at 2 mL min^{-1} by regulating the vacuum pump (5 inHg). Close the cartridge and add 1 mL of hexane–ethyl acetate (7 : 3, v/v). Attach the C_8 cartridge to the top of the silica gel cartridge with a reducing adapter. Add 3 mL of hexane–ethyl acetate (7 : 3, v/v) to the C_8 cartridge. Open the connected cartridges and allow a few drops to drip from the C_8 cartridge into the silica gel cartridge before applying vacuum. This will help to prevent the silica gel cartridge from going dry. When the first 3 mL have reached the top of the C_8 cartridge packing, add an additional 6 mL of hexane–ethyl acetate (7 : 3, v/v). Allow the C_8 eluate to reach the top of the silica gel cartridge packing. Remove the C_8 cartridge and discard. Wash the silica gel cartridge with 3 mL of hexane–ethyl acetate (7 : 3, v/v). Elute and collect the analytes from the silica gel cartridge with 6 mL of ethyl acetate in a 13-mL glass centrifuge tube. Discard the silica gel cartridge. Evaporate the eluate under a slow nitrogen stream (just enough to produce a ripple on the surface) in a water-bath (45 °C) to near dryness (until a thin oily film remains; do not overdry).

6.5 Derivatization (silylation of 3-hydroxymethyl sulfentrazone)

Add 0.5 mL of acetonitrile and 100 μL of fresh BSTFA (Precaution: once the ampule containing BSTFA is opened, the contents should be used within 10 min, since BSTFA will absorb moisture) to the centrifuge tube containing the sample extract, stopper the tube and vortex the sample for 15 s. Add 9.5 mL of hexane–ethyl acetate (9 : 1, v/v) to make 10 mL. Cap the centrifuge tube and vortex the sample until the contents are mixed (there should be no phase separation). If there is a phase separation, gently warm the samples in a water-bath (45 °C) for 1 min. Vortex the sample again. If phase separation persists, continue warming and vortexing the sample until the phases mix.

6.6 Second (post-derivatization) silica gel SPE cartridge

Place a silica gel cartridge (1-g, J.T. Baker) on a vacuum manifold and condition the cartridge with 6 mL of ethyl acetate followed by 6 mL of hexane. Do not allow the

cartridge to go dry at any time during this step. Maintain the flow rate through the silica gel cartridge at about 2 mL min^{-1} by regulating the vacuum pump (5 inHg). Load the derivatized sample extract on to the cartridge. Rinse the centrifuge tube twice, each with 3 mL of hexane–ethyl acetate (9 : 1, v/v), and add the rinsate to the cartridge. Drain the rinsate to the top of the silica gel packing. Elute the analytes with 9 mL of ethyl acetate–hexane (1 : 1, v/v) into a 13-mL glass centrifuge tube. Discard the silica gel cartridge. Add 1 mL of acetonitrile to the eluate. Evaporate the eluate under a slow nitrogen stream (just enough to produce a ripple on the surface) in a water-bath (45 °C) to near dryness (until a thin oily film remains; do not overdry). Dilute the sample to the appropriate final volume with acetonitrile.

6.7 *Analytical procedures for oily crop matrices*

When analyzing oily crop matrices, e.g., sunflower seed and peanut nutmeat, the above method for nonoil crop matrices needs to be slightly modified. Sample extracts of the oily crop matrices need additional hexane and acetonitrile partitions prior to the C_8 SPE cartridge. After acid reflux some of the sulfentrazone compounds tend to adsorb on the oil drops which would not pass through the C_8 SPE cartridge. After the second reflux with 1 N HCl, the entire sample extract is filtered, diluted and partitioned with hexane. The hexane fraction is then partitioned with acetonitrile. The hexane is discarded, the acetonitrile is concentrated to near dryness, and the container is used to collect the eluate from the C_8 SPE cartridge in Section 6.4. The aqueous solution is then passed through the C_8 SPE cartridge; the rest of the analytical procedures are followed as described in Section 6.4.

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6.8 *Analytical procedures for crop refined oils*

Crop refined oils should be dissolved in hexane and extracted in a separatory funnel with 0.25 N HCl follow by an evaporation of residual hexane. Concentrated HCl is then added to make the solution 1 N and the samples are boiled under reflux for 2 h. The rest of the analytical procedures are followed as described in Section 6.4.

6.9 *Instrumentation*

GC was used to analyze the sample extracts. Three detection systems were used, two for quantitation and one for analyte confirmation.

Gas chromatography/electrolytic conductivity detection (GC/ELCD) and gas chromatography/halogen-specific detection (GC/XSD) are specific for halogenated compounds and were effective for discriminating between sulfentrazone compounds and the matrix components. Operating conditions are listed below.

GC/ELCD instrument parameters

Instrument	HP 6890 gas chromatograph
Column	DB-35, 35% phenylmethylsilicone, 30 m × 0.54-mm i.d., 1.0-μm film thickness
Inlet	Splitless injection mode (cyclo-double gooseneck insert)
Detector	O I Analytical 5220 electrolytic conductivity detector, halogen mode
Temperatures	
Injection port	250 °C
Oven	180 °C/1 min (initial), 20 °C min^{-1} (ramp), 260 °C/2 min (hold), 5 °C min^{-1} (ramp), 280 °C/4 min (final)
Reactor	900 °C
Column gas flow rate	He carrier gas, 16 mL min^{-1}
	% 1-Propanol flow 37%
ELCD gas flow rates	H_2 + carrier gas, unvented 135 mL min^{-1}
	H_2 + carrier gas, vented 85 mL min^{-1}
Injection volume	2 μL

GC/XSD instrument parameters

Instrument	HP 6890 gas chromatograph
Column	DB-17, (50% phenyl)silicone, 30 m × 0.546-mm i.d., 1.0-μm film thickness
Inlet	Splitless injection mode (cyclo-double gooseneck insert)
Detector	O I Analytical 5360 halogen-specific detector
Temperatures	
Injection port	250 °C
Oven	180 °C/1 min (initial), 10 °C min^{-1} (ramp), 260 °C/2 min (hold), 5 °C min^{-1} (ramp), 280 °C/5 min (final)
Reactor	1100 °C
Column gas flow rate	He carrier gas, 16 mL min^{-1}
XSD make-up flow rate	Air, 25 mL min^{-1}
Injection volume	2 μL

Operating conditions for spectral analyte confirmation

Instrument	HP 5890 or 6890 gas chromatograph
Column	DB-5MS, 5% phenylmethylsilicone, 15 m × 0.25-mm i.d., 0.25-μm film thickness
Inlet	Splitless injection mode (cyclo-double gooseneck insert)
Detector	HP 5970 or 5972 mass-selective detector
Temperature	
Injection port	260 °C
Oven	120 °C/2 min (initial), 20 °C min^{-1} (ramp), 280 °C/6 min (final)
Detector	280 °C
Gas flow rate	He carrier gas, 1 mL min^{-1}
Injection volume	2 μL

7 Method validation and quality control

7.1 Experimental design

The LOQ was validated by acceptable and reproducible recoveries of the respective analytes from laboratory-fortified control samples. For hay and straw, the LOQ was validated at 0.05 mg kg^{-1} and the LOD was set at 0.01 mg kg^{-1}. For all other matrices, the LOQ was validated at 0.025 mg kg^{-1} and the LOD was set at 0.005 mg kg^{-1}. Each analysis set contained a minimum of one control sample, one fortified control sample, and several treated samples.

A calibration curve was generated for each analyte at the initiation of the analytical phase of the study. Standard solutions for injection contained sulfentrazone, DMS and/or derivatized HMS. Standard solutions were injected at the beginning of each set of assays and after every two or three samples thereafter to gage the instrument response.

7.2 Preparation of standards

Stock solutions of approximately 1 mg mL^{-1} were prepared by dissolving the appropriate amounts of the analytical standards in acetonitrile. Working standard solutions for fortification were prepared in volumetric flasks by appropriate dilutions of the stock solutions for each analyte or combination of analytes. During analysis, SCA is converted to DMS and HMS is derivatized; therefore, the analytical standard solutions for quantitation and instrument calibration contained sulfentrazone, DMS and derivatized HMS. A measured volume of a standard solution containing sulfentrazone, DMS and HMS (prepared from stock solutions) was derivatized simultaneously with the samples.

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7.3 Calculation

The amounts of sulfentrazone, SCA (analyzed as DMS), and HMS were quantitated by an external standard calibration method. A computer spreadsheet program (Microsoft Excel) was used for calculation and reporting.

The amount of sample injected was determined by the following equation:

$$\text{Amount of sample injected (mg)} = \frac{\text{initial aliquot weight (mg)}}{\text{final sample extract volume (}\mu\text{L)}} \times \text{sample extract volume injected (}\mu\text{L)}$$

An equation representing area versus concentration was determined using a standard linear regression analysis applied to the injection standards yielding a slope m and an intercept b. The following equation was then used to calculate the concentration of

the sample injected from the area measured:

$$\text{Concentration of sample (ng } \mu\text{L}^{-1}) = \frac{\text{area of sample} - b}{m}$$

The amount of analyte (in nanograms) detected in a sample injection was calculated by multiplying the concentration calculated above by the injection volume. Then the concentration detected (in ppm) was determined by dividing this result by the amount

Table 1 Recoveries from fortified samples

Matrix	Fortification levels (mg kg^{-1})	No. of analyses	% Recovery (average ± SD)		
			Sulfentrazone	SCA	HMS
Soybean seed	0.025	4	80 ± 7	NA	82 ± 14
Soybean hulls	0.025	1	75	NA	80
Soybean meal	0.025	1	76	NA	70
Soybean refined oil	0.025	3	73 ± 8	NA	90 ± 14
Corn grain	0.025	3	91 ± 11	87 ± 1	79 ± 2
Corn forage	0.025	2	85	82	76
Corn fodder	0.025, 0.05	3	87 ± 9	75 ± 6	76 ± 3
Corn flour	0.025	1	99	102	98
Rice grain	0.025, 0.05	3	104 ± 10	97 ± 18	89 ± 3
Rice straw	0.05, 0.5	2	98	125	86
Rice hulls	0.025	1	95	99	98
Rice bran	0.025	1	77	69	72
Rice, polished	0.025	1	118	72	81
Sorghum grain	0.025	2	95	82	95
Sorghum forage	0.025, 0.05	2	89	86	96
Sorghum fodder	0.025, 0.1	2	84	76	73
Wheat grain	0.025	2	96	120	83
Wheat forage	0.025, 0.1	2	91	89	83
Wheat hay	0.05, 0.2	2	88	89	85
Wheat straw	0.05, 0.5	2	87	114	102
Pea	0.025	4	93 ± 18	70 ± 9	87 ± 12
Alfalfa forage	0.0125, 0.025, 0.05, 0.25	10	105 ± 17	93 ± 15	90 ± 13
Alfalfa hay	0.025, 0.25, 0.2, 1.0	10	75 ± 9	82 ± 12	85 ± 15
Sunflower seed	0.05, 0.5	8	77 ± 14	86 ± 15	89 ± 15
Sunflower meal	0.05, 0.5	6	84 ± 6	73 ± 9	74 ± 5
Sunflower refined oil	0.05, 0.5	6	90 ± 5	103 ± 8	77 ± 4
Sugarcane	0.025, 0.05	5	82 ± 3	70 ± 6	74 ± 3
Refined sugar	0.025, 0.05	2	91	99	87
Molasses	0.025, 0.05	2	94	96	95
Peanut	0.025, 0.05, 0.1, 0.2	7	68 ± 5	77 ± 5	84 ± 7
Peanut meal	0.025	1	68	64	74
Peanut refined oil	0.025, 0.05	3	100 ± 11	86 ± 10	84 ± 7
Potato	0.05, 0.5, 1.0	16	100 ± 7	106 ± 11	87 ± 11
Potato flakes	0.05	1	76	120	84
Potato wet peels	0.25, 0.5	2	121	91	93
Potato chips	0.05, 0.25, 0.5	3	99 ± 4	97 ± 19	82 ± 16

of sample injected:

$$\text{Detected or uncorrected ppm (ng mg}^{-1}\text{)} = \frac{\text{conc. of sample (ng } \mu\text{L}^{-1}\text{)} \times \text{inj. volume } (\mu\text{L})}{\text{amount of sample injected (mg)}}$$

No correction for molecular weights was necessary for derivatized HMS, because the injection standards were derivatized simultaneously with the samples. However, a correction factor was needed for calculating the recovered amount of SCA since the SCA was quantitated as DMS. The correction factor (molecular weight ratio) between SCA and DMS was 1.12 (417/373; 417 = molecular weight of SCA and 373 = molecular weight of DMS). To calculate the amount of SCA, use the above equation, which will yield DMS (ng), then multiply that value by 1.12 to convert to nanograms of SCA.

The uncorrected ppm of the fortified control samples was divided by the fortification level and multiplied by 100% to calculate the method recovery (%). The following equation was used:

$$\text{Method recovery (\%)} = \frac{\text{uncorrected mg kg}^{-1} - \text{control mg kg}^{-1}}{\text{fortification level (mg kg}^{-1}\text{)}} \times 100$$

The LOD was calculated as the concentration of analyte (ppm equivalent) at one-fifth the area of the LOQ level standard, or one-fifth the LOQ, whichever was larger.

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7.4 Time required for analysis

For a set of 10 samples, the analytical method can be completed within 16 laboratory hours from the time of sample weighing to GC injection.

7.5 Accuracy and precision

The accuracy and precision of the analytical methods were determined by the average and standard deviation of individual method recoveries of the fortified-control samples in 40 different matrices (see Table 1). These methods were also demonstrated to be very rugged based on the results of accuracy and precision for a variety of crop matrices.

8 Important points

After the initial extraction with acetone–0.25 N HCl, all traces of acetone must be removed using a TurboVap Evaporator. Traces of solvent can lead to analyte loss through the SPE cartridge(s).

The proper elution and wash solvent composition and the volume and flow rate through the cartridges must be determined. The SPE steps are critical to the separation and cleanup of the sample extract. Listed brands for C_8 and silica gel cartridges should be used, if possible.

After passing the sample solution through the C_8 cartridge, the cartridge and manifold must be completely dry. Extend the drying time if necessary. Rinsing the manifold with acetone prior to elution is a good practice. Traces of aqueous solution may interfere with subsequent derivatization.

BSTFA should be used within 10 min after opening the ampule to ensure complete derivatization. BSTFA readily absorbs moisture, which will interfere with derivatization.

If final sample solutions will be stored for several days, the derivatization of the HMS metabolite may reverse. If the derivatization has reversed, the HMS method recovery would be low and an additional broad peak (underivatized HMS) would be visible after the derivatized HMS peak. In this case, add 10 μL of fresh BSTFA to the final sample solution in the GC vial, vortex the sample for several seconds and re-inject the sample solution.

Optimizing the GC instrument is crucial for the quantitation of sulfentrazone and its metabolites. Before actual analysis, the temperatures, gas flow rates, and the glass insert liner should be optimized. The injection standards must have a low relative standard deviation (<15%) and the calibration standards must have a correlation coefficient of at least 0.99. Before injection of the analysis set, the column should be conditioned with a sample matrix. This can be done by injecting a matrix sample extract several times before the standard, repeating this 'conditioning' until the injection standard gives a reproducible response and provides adequate sensitivity.

Operation of the ELCD and XSD instruments must be optimized for greatest sensitivity. Operating the ELCD instrument in tandem with another detector may cause a decrease in sensitivity. More recently, liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) have been evaluated as possible alternative methods for sulfentrazone compounds in crop matrices. The LC/MS methods allow the chemical derivatization step to be avoided, reducing the analysis time. However, the final sample extracts, after being cleaned up extensively using three SPE cartridges, still exhibited ionization suppression due to the matrix background. Acceptable method recoveries (70–120%) of sulfentrazone compounds have not yet been obtained.

9 Storage stability

Storage stability studies for sulfentrazone compounds on crop matrices showed a pattern of stability for at least 3–38 months, depending on the study program or the maximum sample storage interval for the study.

Acknowledgments

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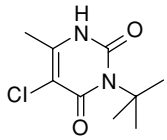
Andrey Chen
FMC, Princeton, NJ, USA

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Terbacil

Material to be analyzed	Alfalfa, apples, blueberries, mint, peaches, strawberries and sugarcane, their processed fractions, and soil
Instrumentation	Gas-chromatographic determination

1 Introduction

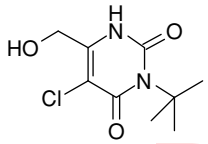
Chemical name (IUPAC)	3-*tert*-Butyl-5-chloro-6-methyluracil
Structural formula	
Empirical formula	$C_9H_{13}ClN_2O_2$
Molar mass	216.7
Melting point	175–177 °C
Boiling point	Sublimation begins below the melting point
Vapor pressure	0.0625 mPa (29.5 °C)
Solubility	Water 710 mg L^{-1} at 25 °C Soluble in polar organic solvents
Stability	Very stable, even at the m.p. Stable in aqueous alkali
Other properties	pK_a 9.5
Use pattern	Terbacil is a substituted uracil herbicide used primarily to control annual and perennial grass and broadleaved weeds in apple orchards, alfalfa, asparagus, mint and sugar cane. Other minor uses include blueberries, strawberries and seed grasses
Regulatory position	The residue definition includes terbacil and its three primary metabolites, Metabolite A, Metabolite B and Metabolite C

2 Outline of method

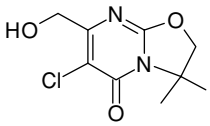
The current residue method for the determination of terbacil and its metabolites in agricultural commodities follows the 'Pesticide Analytical Manual', Volume II,[1]

Handbook of Residue Analytical Methods for Agrochemicals.
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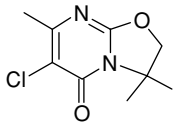
with two additional steps to improve the stability of the analytes and the purity of the final extract. Terbacil is extracted from plant materials with chloroform. After addition of a small volume of water, the extract is concentrated to ~5 mL. The residue is reconstituted in acetonitrile and partitioned with hexane to remove impurities. The resultant acetonitrile layer is concentrated to dryness and reconstituted in basic aqueous solution. Terbacil is then extracted into ethyl acetate by repeated partition of the aqueous base. The combined ethyl acetate extracts are filtered through a bed of anhydrous sodium sulfate and concentrated to 1 mL. Derivatization of the extract (for analysis of metabolites) is carried out by the addition of bis(trimethylsilyl) trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS), allowing the reaction mixture to stand at room temperature overnight or for 16 h. Calibration standards are concurrently derivatized under the same conditions. Water is added to the derivatization reaction mixture and the analytes are partitioned into ethyl acetate. The derivatized analytes are passed through a Florisil SPE cartridge followed by gas chromatography/nitrogen–phosphorus detection (GC/NPD) analysis. Terbacil and its Metabolites A, B and C, can be determined simultaneously by this method.



Metabolite A



Metabolite B



Metabolite C

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3 Apparatus

Disposable culture tubes, 16 × 125-mm
Florisil Sep-Pak, Waters (Milford, MA, USA), Part No. 51960
Waring blender and blending cups, stainless steel or glass
Round-bottom flasks, assorted sizes
Cotton batting
Separatory funnels, 250-mL
Long-stem filtering funnels
Derivatizing vials, 10-mL
Volumetric flasks, various sizes
Volumetric pipettes, 1- and 4-mL
Bond Elut reservoirs and adapters, Analytichem International (Harbor City, CA, USA)
Vacuum filtration adapter, Aldrich (Milwaukee, WI, USA), Cat. No. Z11,562-2
Rubber adapters
Vacuum evaporator, Büchi Model RE121, Brinkmann Instruments, Inc. (Burlingame, CA, USA), with temperature-controlled water-bath
Nitrogen evaporator, Meyer Model 111, Organomation Associates, Inc. (South Berlin, MA, USA), with temperature-controlled water-bath

Glass-wool
Amber-colored bottles with Teflon-lined lids

4 Reagents

Acetonitrile, HPLC grade
BSTFA plus 1% TMCS, Pierce (Rockford, IL, USA)
Chloroform, HPLC grade
Ethyl acetate, HPLC grade
n-Hexane, HPLC grade
Methanol, HPLC grade
Sodium hydroxide
Sodium sulfate, anhydrous
Toluene, HPLC grade
Water, HPLC grade

5 Sampling and preparation

Matrix samples, where appropriate, are processed by grinding with dry-ice either in a food processor or a Hobart–Cutter mixer and stored in zipper-locking storage bags, allowing the dry-ice to sublime. All processed samples are then stored frozen ($<0\,^{\circ}C$) until used for fortification and analysis.

6 Procedure

6.1 Extraction

All processed samples are removed from the freezer and allowed to reach room temperature. Weigh 10 g of a representative sample into the blender cup and add 150 mL of chloroform. Blend the sample for 5 min. Pass the chloroform extract through a cotton-plugged funnel into a 1000-mL round-bottom flask. Extract the matrix twice more with 100-mL portions of chloroform and filter the extracts through the cotton. Rinse the blender cup and cap with chloroform until all particulates have been removed from the cup. Add 10 mL of water to the combined extracts and concentrate the chloroform with a rotary evaporator at 35 °C. Transfer the residue (ca 5 mL of water with sonication) using several volumes of acetonitrile into a 250-mL separatory funnel (the final volume should be less than 100 mL). Add 50 mL of hexane and shake the funnel for 1 min. Allow the phases to separate. Discard the hexane and repeat the partitioning with two additional portions of hexane. Quantitatively transfer the acetonitrile into a 250-mL round-bottom flask and roto-evaporate the extract to dryness at 35 °C. Dissolve the dry residue using several rinses of 0.1% NaOH and transfer the solution into a 250-mL separatory funnel (the final volume should be less than 80 mL). Add 75 mL of ethyl acetate and shake the funnel for 2 min. Allow the phases to separate. Filter the ethyl acetate through a 4-cm bed of anhydrous sodium

sulfate into a 500-mL round-bottom flask. Repeat the partitioning with ethyl acetate three more times and combine the ethyl acetate extracts. Concentrate the combined extracts to ca 5 mL by roto-evaporation at 35 °C.

6.2 Derivatization

Quantitatively transfer the concentrate from the 500-mL round-bottom flask into a 10-mL 'derivatizing' vial with additional rinses of ethyl acetate. Concentrate the sample to 1 mL under a gentle stream of nitrogen at 35 °C. Add 300 µL of BSTFA + 1% TMCS to the derivatizing vial, cap the vial with a Teflon-lined lid and shake the vial vigorously for approximately 20 s. Allow the derivatization to take place overnight or for at least 16 h at room temperature. Simultaneously with sample derivatization, prepare a 20 µg mL^{-1} standard of all analytes by pipetting 4 mL of the 5 µg mL^{-1} standard solutions used for fortification into a 10-mL derivatization vial. Concentrate the standard solution to 1 mL under nitrogen and derivatize the standard along with the samples.

6.3 Cleanup

Prepare a sodium sulfate mini-column for each sample and standard by plugging the bottom of a 12.5-cm Pasteur pipette with a small amount of glass-wool and filling the pipette with anhydrous sodium sulfate until it is approximately one-third full. Place the mini-columns into 16 × 125 mm disposable culture tubes. After at least 16 h of derivatization, add 1 mL of deionized water to the samples and shake each vial for 20 s. Allow the phases to separate. Do not add water to the standards until after completion of the final sample cleanup to ensure stability of the standards. Remove the upper phase from the derivatizing vial with a Pasteur pipette and place the extract on the top of the sodium sulfate mini-column. Rinse the column twice with 2 mL of ethyl acetate and collect the eluate in the culture tube. Concentrate the solution to dryness under nitrogen at 35 °C and resuspend the dry residue in 10 mL of ethyl acetate–hexane (1 : 4, v/v). Dissolve the dry residue by repeated rinsing of the tube walls with a Pasteur pipette. Attach an Analytichem reservoir to a Waters 900-mg Florisil Sep-Pak connected to a vacuum adapter joined to a 250-mL round-bottom flask and attach the column to a light vacuum. Pre-rinse the Florisil Sep-Pak with 5 mL of ethyl acetate–hexane (1 : 4, v/v) solvent mixture and add the 10 mL of sample to the reservoir. Allow the sample to elute through the Florisil cartridge at approximately 10 mL min^{-1}. Rinse the sample tube with 5 mL of the same ethyl acetate–hexane solvent mixture and transfer the solvent to the reservoir. Rinse the culture tube with 5 mL of methanol–ethyl acetate–toluene (2 : 1 : 17, v/v/v) solvent and draw the solvent through the cartridge, collecting all fractions into the 250-mL round-bottom flask.

Concentrate the extract to dryness by roto-evaporation at 35 °C and resuspend the dry residue in 3 mL of ethyl acetate. Transfer all the extract into a clean culture tube and rinse the flask with several small portions of ethyl acetate. Transfer the rinsings to the culture tube. Add water to the standard and dry using the sodium sulfate

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mini-column as described above. Concentrate both the samples and the standard to dryness under nitrogen at 35 °C and suspend all sample extracts and standards in 1 mL of ethyl acetate. Dissolve the dry residue by repeated rinsing of the tube walls with a Pasteur pipette.

Transfer all samples and calibrant standards to gas chromatography (GC) vials. Inject samples on to the GC column in the following order: 10 μg mL^{-1} standard, sample, sample, 5 μg mL^{-1} standard, sample, sample, 2 μg mL^{-1} standard, etc.

6.4 Determination

Instrumentation	Hewlett-Packard Model 5890 gas chromatograph equipped with a nitrogen–phosphorus detector Hewlett-Packard Model 3396 or 3396A integrator Hewlett-Packard Model 6890 or 7673A autosampler	
Column	RTX-1 fused-silica column (100% polymethylsiloxane), 30 m × 0.53-mm i.d., 0.5-μm film thickness, Restek Corporation (Bellefonte, PA, USA)	
Injector temperature	275 °C	
Detector temperature	300 °C	
Oven temperature	Initial temperature	90 °C for 1 min
	First ramp	90 to 185 °C at 5 °C min^{-1}
	Second ramp	185 to 260 °C at 30 °C min^{-1}
	Final hold	260 °C for 5 min
Gases	Helium carrier gas, 8 mL min^{-1} Helium make-up gas, 30 mL min^{-1} Hydrogen, 4 mL min^{-1} Air, 100 mL min^{-1}	
Injection volume	2 μL	

GC retention times for terbacil and its metabolites were dependent on the oven temperature and gas flow rates. Approximate retention times for the analytes were 13.1, 15.8, 18.4 and 19.4 min for Metabolite C, terbacil, Metabolite A and Metabolite B, respectively.

7 Evaluation

7.1 Method

Concentrations of terbacil and its Metabolites A, B and C are calculated from a calibration curve for each analyte run concurrently with each sample set. The equation of the line based on the peak height of the standard versus nanograms injected is generated by least-squares linear regression analysis performed using Microsoft Excel.

7.2 Recoveries, limit of detection and limit of determination

The recoveries of terbacil from untreated control plant matrices fortified at levels from 0.10 to 1.0 mg kg^{-1} range from 85 to 130%. The recoveries of Metabolites A, B and C range from 80 to 130, 50 to 100 and 90 to 110%, respectively. The limit of detection for terbacil is 0.5 mg kg^{-1}, with a limit of determination of 0.05 mg kg^{-1}.

7.3 Calculation of residues

The equation for calculation of the total concentration of terbacil and its metabolites is as follows:

$$\text{Linear regression equation from calibration curve } y = mx + b$$

where

y = peak area
m = calibration slope
x = amount injected (ng)
b = calibration intercept

$$\text{Concentration of analyte} = \frac{\text{ng injected}}{\text{mg matrix injected}} \times \text{dilution factor}$$

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where

$$\text{mg matrix injected} = \frac{\text{weight of sample (10 g)}}{\text{sample vol. (1 mL)}} \times \text{injection volume (0.002 mL)}$$
$$= 20 \text{ mg}$$

$$\text{Recovery (\%)} = \frac{\text{concentration in fortified matrix } (\mu g\, g^{-1}) - \text{concentration in control } (\mu g\, g^{-1})}{\text{concentration fortified } (\mu g\, g^{-1})} \times 100$$

8 Important points

Two steps have been added to the original 'Pesticide Analytical Manual' method to increase the stability of the trimethylsilyl derivatives and to clean up the final extract prior to GC analysis, namely the use of a sodium sulfate mini-column to dry the extract after derivatization and the use of a Florisil Sep-Pak cartridge to remove matrix interferences. The advantages of the current method are the simultaneous evaluation of the four analytes, reproducibility and low matrix interference.

Reference

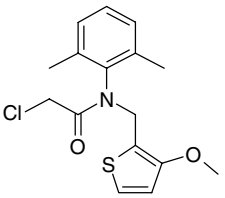
1. 'Pesticide Analytical Manual', US Department of Health and Human Services, Washington, DC, Vol. II, pp. 180–209 (1994).

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Thenylchlor

Materials to be analyzed	Rice grain, soil and water
Instrumentation	Gas-chromatographic determination for rice grain, water and soil

1 Introduction

Chemical name (IUPAC)	2-Chloro-*N*-(3-methoxy-2-thenyl)-2′,6′-dimethyl-acetanilide
Structural formula	
Empirical formula	$C_{16}H_{18}ClNO_2S$
Molar mass	323.8
Melting point	72–74 °C
Boiling point	173–175 °C/0.5 mmHg
Vapor pressure	2.8×10^{-2} mPa at 25 °C
Solubility	Water 11 mg L^{-1} at 20 °C
Stability	Decomposition at 260 °C and by ultraviolet (UV) irradiation (400 nm, 8 h). Stable to acid and base in the pH range 3–8.
Use pattern	Thenylchlor is a chloroacetamide herbicide for the pre-emergence and post-planting control of annual grass and broad-leaved weeds in paddy rice
Regulatory position	The residue definition is for the parent, thenylchlor, only

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2 Outline of method

Milled rice grain is soaked with water and allowed to stand at room temperature for 2 h. The soaked sample is mixed with acetone. The extract is filtered, the acetone in the extract is evaporated and the aqueous residue is transferred into a C_{18} cartridge.

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The eluate from the C_{18} cartridge is concentrated by rotary evaporation and the residue is dissolved in n-hexane and then subjected to a cleanup procedure using a Florisil cartridge. The eluate is dried and analyzed by gas chromatography (GC) with nitrogen–phosphorus detection (NPD).

3 Apparatus

High-speed blender fitted with a leak-proof glass jar and explosion-proof motor
Laboratory mechanical shaker
Filter paper, 6-cm diameter (Kiriyama 5A filter paper)
Glass filter paper, 6-cm diameter
Erlenmeyer flask, 300-mL
Filtration flask, 300-mL
Round-bottom flasks, 100-, 300- and 500-mL
Rotary vacuum evaporator, 40 °C bath temperature
Separatory funnel, 500-mL
Gas chromatograph equipped with a nitrogen–phosphorus detector
Water-bath: electrically heated, temperature 45 °C

4 Reagents

Acetonitrile, n-hexane, acetone, methanol and dichloromethane, reagent grade
Sodium chloride
Sodium sulfate, anhydrous
C_{18} cartridge: Bond Elut, 1-g/6-mL (Varian)
Florisil cartridge: Sep-Pak Plus Florisil cartridge (Waters)
Silica gel: Wako gel C-200, 100–200 mesh
Alumina cartridge: Sep-Pak Plus Alumina N cartridge (Waters)
Nitrogen, repurified
Hydrogen, repurified

5 Sampling and preparation

Mill the unpolished rice grain with an ultracentrifuge mill in the presence of dry-ice and sieve through a 42-mesh screen.

6 Procedure

6.1 Extraction

6.1.1 Rice grain

Weigh 10 g of the milled sample in a 300-mL Erlenmeyer flask and soak in 20 mL of distilled water for 2 h. After adding 100 mL of acetone and shaking vigorously with a mechanical shaker for 30 min, filter the extract by suction through overlapping

filter paper and glass filter paper. Add 50 mL of acetone to the residue, shake and filter the extract as before. Combine the filtrates in a 300-mL round-bottom flask and concentrate by rotary evaporation.

6.1.2 Soil

Mix 10 g of the air-dried soil with 100 mL of acetone and shake the mixture with a mechanical shaker for 30 min. Filter the mixture through a fluted filter paper into a 300-mL round-bottom flask. Wash the residue on the filter with 50 mL of acetone. Combine the filtrates and concentrate by rotary evaporation.

6.1.3 Water

Transfer 200 mL of water into a 500-mL separatory funnel. Extract the sample twice with 50 mL of dichloromethane for 30 min with a mechanical shaker and collect the extracts in a 300-mL Erlenmeyer flask. Filter the combined extracts, together with the washings from the collecting flask, through anhydrous sodium sulfate into a 300-mL round-bottom flask. Remove the dichloromethane by rotary evaporation. Dissolve the residue in 10 mL of n-hexane.

6.2 Cleanup

6.2.1 Rice grain

First cleanup: Transfer the concentrate into a C_{18} cartridge preconditioned with 5 mL of methanol, followed by 5 mL of water. Rinse the cartridge with 5 mL of acetonitrile–water (3 : 7, v/v). Elute thenylchlor with 10 mL of acetonitrile. Concentrate the eluate to dryness by rotary evaporation at 40 °C. Dissolve the residue in 5 mL of n-hexane.

Second cleanup: Transfer the n-hexane solution into a Florisil cartridge preconditioned with 5 mL of n-hexane and elute thenylchlor with 10 mL of acetone–n-hexane (1 : 9, v/v). Concentrate the eluate to dryness by rotary evaporation at 40 °C and dissolve the residue in 5 ml of acetone for GC analysis.

6.2.2 Soil

First cleanup: Transfer the concentrate into a C_{18} cartridge preconditioned with 5 mL of methanol, followed by 5 mL of water. Rinse the cartridge with 5 mL of acetonitrile–water (3 : 7, v/v). Elute thenylchlor with 10 mL of acetonitrile. Concentrate the eluate to dryness by rotary evaporation at 40 °C. Dissolve the residue in 5 mL of n-hexane.

Second cleanup: Transfer the n-hexane solution into an alumina cartridge preconditioned with 5 mL of n-hexane. Rinse the cartridge with 10 mL of acetone–n-hexane (1 : 9, v/v) and elute thenylchlor with 7 mL of acetone–n-hexane (3 : 17, v/v). Concentrate the eluate to dryness by rotary evaporation at 40 °C and dissolve the residue in 5 mL of acetone for GC analysis.

6.2.3 Water

Transfer the n-hexane solution into a glass column packed with 5 g of Florisil saturated with n-hexane. Rinse the column, first with 2 mL of n-hexane and then with 50 mL

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of acetone–n-hexane (1 : 19, v/v). Elute thenylchlor with 50 mL of acetone–n-hexane (1 : 9, v/v). Concentrate the eluate to dryness by rotary evaporation at 40 °C and dissolve the residue in 5 mL of acetone for GC analysis.

6.3 Determination (rice grain, soil and water)

Inject an aliquot of the solution into the gas chromatograph.

Operating conditions

Gas chromatograph	HP 5890A, Hewlett-Packard
Sample injector	Solventless sample injector
Column	DB-1 (J&W Scientific), 0.53-mm i.d. × 15 m, film thickness 1.5-μm
Column temperature	235 °C
Injection port temperature	250 °C
Detector	Nitrogen–phosphorus detector
Detector temperature	280 °C
Gas flow rates	Helium carrier gas, 10 mL min^{-1} Hydrogen, 3.5 mL min^{-1} Air, 115 mL min^{-1}
Injection volume	2 μL
Retention time of thenylchlor	4.5 min

7 Evaluation

7.1 Method

7.1.1 Rice grain and soil

Quantitation is performed by the calibration technique. Construct a new calibration curve with thenylchlor standard solutions for each set of analyses. The thenylchlor peak usually appears at a retention time around 4.5 min. Plot the peak area against the injected amount of thenylchlor. The injection volume (2 μL) should be kept constant as the peak area varies with the injection volume with NPD. Before injecting the sample solutions, check the stability of sensitivity of the GC system by injecting more than one standard solution containing ca 0.05–2 ng of thenylchlor. Recommendation: inject standard solutions and sample solutions alternately rather than constructing the calibration curve in advance.

7.2 Recoveries and limit of detection

7.2.1 Rice grain

The recoveries from untreated control samples fortified with thenylchlor at levels of 0.2 mg kg^{-1} ranged from 75 to 100%. The limit of detection was 0.01 mg kg^{-1}.

7.2.2 Soil

The recoveries from untreated control soil fortified with thenylchlor at levels between 0.3 and 3.0 mg kg^{-1} ranged from 91 to 95%. The limit of detection was 0.01 mg kg^{-1}.

7.3 Calculation of residues

The amount of thenylchlor residue (R, mg kg^{-1}) in the sample is calculated using the following equation:

$$R = (W_i \times V_f)/(V_i \times G)$$

where

G = sample weight (g)
V_i = injection volume into the gas chromatograph (μL)
V_f = final sample volume (mL)
W_i = amount of thenylchlor for V_i read from the calibration curve (ng)

8 Important points

To avoid degradation of the analytes, rice grain samples must be homogenized and milled in the presence of dry-ice. During evaporation of organic solvents, the temperature of the water-bath should be kept at 40 °C or lower.

Thenylchlor residues in rice grain fortified with thenylchlor at levels of 0.5 mg kg^{-1} are stable, with more than 95% remaining after approximately 45 days of storage at −20 °C (personal data).

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References

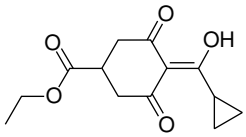
1. 'Persistency in Agricultural Products and Water Pollution of Pesticides,' No. 3, The Chemical Daily Co., Tokyo, pp. 492–494 (1998) (in Japanese).
2. 'Analytical Method for Thenylchlor,' Ministry of the Environment, Tokyo (1993).
3. K. Kobayashi, M. Onoe and H. Sugiyama, *Weed Res. (Jpn),* **39**, 160 (1994).

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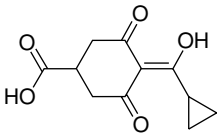
Trinexapac-ethyl

Materials to be analyzed	Soil, air, and water
Instrumentation	High-performance liquid chromatographic system equipped with an automated column switching system

1 Introduction

Trinexapac-ethyl	
Chemical name (IUPAC)	Ethyl 4-cyclopropyl(hydroxy)methylene-3,5-dioxocyclohexanecarboxylate
CAS RN	95266-40-3
Development code	CGA-163935
Chemical class	Carboxylic acid ester
Structural formula	
Empirical formula	$C_{13}H_{16}O_5$
Molar mass	252.3
Melting point	36.1–36.6 °C
Boiling point	<270 °C
pK_a value	4.57 (20 °C)
Vapor pressure	1.6 mPa (20 °C), 2.2 mPa (25 °C)
Solubility (25 °C)	In water, 2.8 (pH 4.9), 10.2 (pH 5.5), 21.1 g L^{-1} (pH 8.2); methanol, acetonitrile, cyclohexanone >1 g mL^{-1}; 2-propanol 0.9 g mL^{-1}, n-octanol 180 mg mL^{-1}, hexane 35 mg mL^{-1}
Stability	Stable in neutral aqueous solution, less stable in basic aqueous solution
Other properties	Trinexapac-ethyl decomposes to its acid form in acidic or basic aqueous solution and is light sensitive. Samples should be analyzed immediately or stored in the dark until analysis

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Use pattern	Trinexapac-ethyl is a plant growth regulator used to prevent lodging in cereals and oilseed rape. Other uses include reduction of turf growth and promotion of early maturation in sugarcane
Regulatory position	The definition of residues of regulatory concern are for the parent trinexapac-ethyl and its acid metabolite, trinexapac
Trinexapac	
Chemical name (IUPAC)	4-Cyclopropyl(hydroxy)methylene-3,5-dioxo-cyclohexanecarboxylic acid
CAS RN	104273-73-6
Development code:	CGA-179500
pK_a value	~3.5
Structural formula	

2 Outline of methods[1–3]

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2.1 Trinexapac-ethyl

A 5-g soil sample is homogenized with 50 mL of methanol–phosphate buffer (pH 7)–water (15 : 7 : 28, v/v/v) solvent mixture. A 10-mL aliquot portion of the supernatant is loaded on to and eluted through a silica-based solid-phase extraction (SPE) cartridge (Clin-Elut 1020). The eluate is evaporated to dryness and the trinexapac-ethyl residue is dissolved in 5 mL of high-performance liquid chromatography (HPLC) injection solution [900 mL of water + 50 mL of phosphate buffer (pH 7) +50 mL of acetonitrile (ACN) + 4 g of tetrabutylammonium bromide (TBABr)] and analyzed by high-performance liquid chromatography/ultraviolet detection (HPLC/UV) with a three-column switching system.

A water sample (500-mL) is acidified to pH 3 with HCl and mixed with 20 mL of saturated NaCl solution. The sample is extracted via liquid/liquid partitioning into methylene chloride (3 × 50 mL). The combined methylene chloride fraction is reduced to dryness via rotary evaporation and the residue reconstituted in water–ACN (3 : 1, v/v). The final determination is performed using HPLC/UV with a two-column switching system.

For air analysis, a known volume of air is passed through a sampling cartridge for a preset period of time. The cartridge is eluted with methanol and reduced to dryness prior to reconstitution of the residue in 10 mL of water–ACN–85% phosphoric acid (700 : 300 : 1, v/v/v) solvent mixture. Residue determination is carried out using HPLC/UV at 280 nm.

2.2 Trinexapac

A 50-g soil sample is homogenized with 200 mL of water (if the solution pH is <6, adjust to pH 6–8 using 1 M NaOH). A 100-mL aliquot portion of the soil/water supernatant is extracted with a 2-g C_{18} cartridge followed by a 5-g C_{18} cartridge and the eluate is evaporated to dryness. The residue of trinexapac is dissolved in 4 mL of water–phosphate buffer (pH 7)–ACN–TBABr (90 : 5 : 5 : 0.3). Residue determination is performed using HPLC/UV with a two-column switching system.

A 200-mL water sample is adjusted to pH 6–8 using 1 M NaOH followed by the addition of 2 mL of methanol. The sample is concentrated and cleaned up using two consecutive cartridges, a 2-g C_{18} cartridge followed by a 1-g C_{18} cartridge. The residue is reconstituted in 4 mL of water–phosphate buffer (pH 7)–ACN–TBABr (90 : 5 : 5 : 0.3) for analysis using HPLC/UV with a two-column switching system.

3 Apparatus

Amber-colored bottles
Concentration tubes
Centrifuge
Disposable syringe filter, 0.2-μm or 0.45-μm
Round-bottom flasks, KIMAX
Glass-wool
Laboratory homogenizer and mechanical shaker
Nitrogen evaporator
pH meter
Rotary evaporators
Separatory funnels
SPE vacuum manifolds
Volumetric pipettes
Vortex mixer
Ultrasonic bath
Teflon vacuum pump
Air sampler, Alpha-1 (Ametek)
Mini-buck calibrator, mode M-5
OSHA versatile sampler (OVS) sorbent tubes

4 Reagents

Acetonitrile (ACN), HPLC grade
Dichloromethane, analytical grade
Ethyl acetate, analytical grade
n-Hexane, analytical grade
Methanol, HPLC grade
Phosphate buffer (pH 7): 0.041 M $Na_2HPO_4 + 0.028$ M KH_2PO_4

Phosphoric acid (H_3PO_4), analytical grade, 85%
Sodium hydroxide (NaOH)
Sodium sulfate, anhydrous
Tetrabutylammonium bromide (TBABr), recrystallize from ethyl acetate
Ultrapure water for HPLC analysis

Preparation of standard solutions

Stock solution. Prepare a 200-μg mL^{-1} stock solution in ACN
Fortification solutions. Prepare 0.04 and 0.2 μg mL^{-1} fortification solutions by dilution of the stock solution in acetonitrile (trinexapac-ethyl). Prepare 0.05 and 0.25 μg mL^{-1} fortification solutions by dilution of the stock solution in water (trinexapac).
Standard solutions. Prepare different concentrations of standard solutions by dilutions of the stock solution with the injection solution.
Injection solutions. Indicated in each of the methods.

5 Sampling and preparation

No specific sample preparation and processing are needed for these methods.

6 Extraction and cleanup

6.1 Trinexapac-ethyl

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6.1.1 Soil

Heat and reflux a 5-g portion of soil sample with 50 mL of methanol–phosphate buffer (pH 7)–water (15 : 7 : 28, v/v/v) solvent mixture in a round-bottom flask for 1 h. After cooling, transfer a 10-mL portion of the supernatant to a test-tube and mix with 11 mL of 0.02 M H_3PO_4 solution. Load this solution on to a silica-based SPE cartridge (Analytichem International Clin-Elut 1020) at a flow rate of 1–2 drops per second. Discard this fraction. Elute the analytes with 30 mL of dichloromethane. Concentrate the eluate to dryness with air in a water-bath at a temperature of 40 °C (do not use vacuum). Dissolve the residues in 5 mL of HPLC injection solution [900 mL of water + 50 mL of phosphate buffer (pH 7) +50 mL of ACN +4 g of TBABr]. Final analysis is performed using liquid chromatography/ultraviolet detection (LC/UV) with a three-column switching system.

6.1.2 Water

Transfer a 500-mL water sample to a 1000-mL separatory funnel, add 20 mL of saturated NaCl solution and adjust the pH to 3 ± 0.1 using either 0.12 N HCl or 0.1 M NaOH. Partition the water sample with 50 mL of dichloromethane. After phase separation, dry the dichloromethane portion through a pad of sodium sulfate (prewashed with 50 mL of dichloromethane). Repeat the partition procedure two additional times, drying each fraction. Concentrate the pooled dichloromethane fractions

to dryness using rotary evaporation at a water-bath temperature of 40 °C. Reconstitute the residue with 0.50 mL of water–ACN (3 : 1, v/v). Residues of trinexapac-ethyl are analyzed using LC/UV with a two-column switching system.

6.1.3 Air

Before sampling, connect an OVS sorbent tube directly to an air sampler using a short piece of Teflon tubing and adjust the air flow rate to 0.5 mL min^{-1}. This rate should be kept constant throughout the sampling period. Pass 120 L of air through the system; terminate sampling by removing and capping the tube. Transfer the Teflon holding ring, glass-fiber filter, and the first XAD-2 layer from the OVS tube to a 10-mL round-bottom flask (Sample 1). Transfer the first polyurethane foam pad and the second XAD-2 layer from the OVS tube to a second 10-mL round-bottom flask (Sample 2). Add 5 mL of methanol to each flask and extract the trapped trinexapac-ethyl from the sorbent into the methanol by ultrasonic extraction for 5 min. Transfer each methanol extract to its respective 25-mL concentration tube and repeat the extraction procedure for each one more time using another 5 mL portion of methanol in each tube. Combine the two methanol fractions for Sample 1 (and for Sample 2) and add 1 drop of diethylene glycol diethyl ether. Evaporate the solvent to dryness using rotary evaporation at a water-bath temperature of 35 °C. Dissolve the residue in 10 mL of ACN–water (3 : 7, v/v, containing 0.1% H_3PO_4) for analysis using LC/UV.

6.2 Trinexapac

6.2.1 Soil

Combine a 50-g soil sample with 200 mL of ultra-pure water in a 500-mL round-bottom flask. Seal and shake the mixture vigorously for 2 min. Measure the pH to ensure that the pH of the mixed solution is between 6 and 8. Connect the flask to a water-cooled condenser (ca 30-cm height) and reflux the mixture for 2 h. Cool the solution and transfer >100 mL of the extract to a centrifuge tube of sufficient size. Centrifuge the extract for 15 min at 3500 rpm. Save a 100-mL aliquot portion of the supernatant for further cleanup. Adjust the pH to 6–8 with phosphate buffer (pH 7) if needed, add 1 mL of methanol to the extract aliquot portion and shake the solution well.

Condition a Varian Mega Bond Elut, 2-g C_{18} SPE cartridge with 10 mL of methanol followed by 10 mL of ultrapure water and load the extract on to the preconditioned C_{18} SPE cartridge. Rinse the glassware that contained the extract aliquot with ca 10 mL of water and transfer the rinse water to the SPE cartridge. Collect the entire sample (the extract aliquot and the wash solution) passing through the SPE cartridge using a beaker placed inside the vacuum manifold. Add 2 mL of 85% H_3PO_4 and 5 mL of methanol to the collected sample solution.

The second cleanup step uses a 5-g Varian Mega Bond Elut C_{18} SPE cartridge, preconditioned with 20 mL of methanol and 20 mL of 85% H_3PO_4–water (1 : 50, v/v). Load the entire volume of the sample solution precleaned by the first SPE cartridge on to the second C_{18} SPE cartridge. Rinse all glassware used for collecting and

transferring the sample extract with 2% aqueous H_3PO_4 solution and add the rinse water to the 5-g C_{18} cartridge. Wash the cartridge with 5 mL of methanol–2% aqueous H_3PO_4 (7 : 3, v/v) and discard. Dry the cartridge under vacuum for about 1 min. Elute the trinexapac residue with 6 mL of methanol. Collect and evaporate the eluate to dryness using a gentle stream of air. Dissolve the residue in 4 mL of 0.3% TBABr in a water–phosphate buffer (pH 7)–ACN (18 : 1 : 1, v/v/v). The residue of trinexapac is subjected to determination by LC/UV with a two-column switching system.

6.2.2 Water

Transfer 200 mL of water sample to a graduated cylinder and measure the pH. Adjust the pH to 6–8 with phosphate buffer (pH 7) if required, add 2 mL of methanol to the water sample, and mix the solution well. Condition a 1-g C_{18} SPE cartridge with 5 mL of methanol followed by 10 mL of ultrapure water. Load the water sample on to the SPE cartridge under gravity. Rinse the glassware that contained the extract aliquot with ca 10 mL of water and transfer the rinse water to the SPE cartridge. Collect the entire sample (the extract aliquot and the wash solution) passing through the SPE cartridge using a beaker placed inside the vacuum manifold. Add 2 mL of 85% H_3PO_4 to the collected sample solution.

Condition a second 1-g C_{18} SPE cartridge with 5 mL of methanol followed by 10 mL of 85% H_3PO_4–water (1 : 100, v/v). Load the sample solution precleaned by the first SPE cartridge on to the second C_{18} SPE cartridge. Rinse all glassware used for collecting and transferring the sample extract with water containing 2% H_3PO_4 and add the rinse solution to the second C_{18} cartridge. Use 2 mL of methanol–water (3 : 7, v/v) to wash the SPE cartridge and dry the cartridge under vacuum for about 5 min. Elute the analyte with two 1.5-mL portions of methanol. Collect the eluate and evaporate it to dryness using a gentle stream of air. Dissolve the residue in 4 mL of 0.3% TBABr in a water–phosphate buffer (pH 7)–ACN (18 : 1 : 1, v/v/v) for analysis using LC/UV with a two-column switching system.

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6.3 Determination

A constant-temperature column oven is essential to ensure constant and reproducible elution times and cutting intervals during each HPLC analysis.

Operating conditions

Soil

An HPLC system equipped with an ultraviolet/diode-array detector (UV/DAD) and automated column switching system is used.

Column 1 (purification)	Hamilton PRP1 porous polymer, 10-μm, 4.6 × 250 mm
Column 2 (purification)	Inertsil phenyl, 5-μm, 4.6 × 250 mm
Column 3 (analysis)	Inertsil C18, 10-μm, 4.6 × 250 mm
Mobile phase 1	Aqueous phosphate buffer (pH 7)–ACN (4 : 1, v/v)
	Preparation of aqueous phosphate buffer (pH 7): phosphate buffer (pH 7) (30 mL) + water (770 mL) + TBABr (4 g)

Mobile phase 2	Aqueous phosphate buffer (pH 2)–ACN (11 : 9, v/v) Preparation of aqueous phosphate buffer (pH 2): 1 M H_3PO_4 (30 mL) + 0.5 M H_2SO_4 (20 mL) + 1 M NaOH (10 mL) + water (490 mL)
Mobile phase 3	Aqueous phosphate buffer (pH 2)–ACN (9 : 11, v/v) Preparation of aqueous phosphate buffer (pH 2): 1 M H_3PO_4 (30 mL) + 0.5 M H_2SO_4 (20 mL) + 1 M NaOH (10 mL) + water (390 mL)
Flow rate 1	1.0 mL min^{-1}
Flow rate 2	1.0 mL min^{-1}
Flow rate 3	1.0 mL min^{-1}
Column switching time 1	14 min
Column switching time 2	25 min (overall)
Retention time	33 min (overall)
Column temperature	25 °C
Detection	ultraviolet (UV) detector at 280 nm
Injection volume	1 mL

Air

An HPLC system equipped with a UV/DAD is used.

Column	Nucleosil 100 C_{18}, 5-μm, 4 × 120 mm
Mobile phase	Water (600 mL) + ACN (400 mL) + 85% H_3PO_4 (1 mL)
Flow rate	1.0 mL min^{-1}
Retention time	ca 6 min
Column temperature	Ambient
Detection	UV detector at 280 nm
Injection volume	100 μL

Water

An HPLC system equipped with a UV/DAD and automated column switching system is used.

Column 1 (purification)	Hamilton PRP1 porous polymer, 10-μm, 4.6 × 250 mm
Column 2 (analytical)	Inertsil ODS, 10-μm, 4.6 × 250 mm
Mobile phase 1	Aqueous phosphate buffer (pH 7)–ACN (21 : 4, v/v) Preparation of aqueous phosphate buffer (pH 7): phosphate buffer (pH 7) (30 mL) + water (810 mL) + TBABr (3 g)
Mobile phase 2	Aqueous phosphate buffer (pH 2)–ACN (3 : 1, v/v) Preparation of aqueous phosphate buffer (pH 2): 1 M H_3PO_4 (30 mL) + 0.5 M H_2SO_4 (20 mL) + 1 M NaOH (10 mL) + water (690 mL) + TBABr (1 g)
Flow rate 1	1.5 mL min^{-1}
Flow rate 2	1.0 mL min^{-1}

Column switching time	17 min (exact switching time is determined by directly connecting the column to the detector)
Retention time	33 min (overall)
Transfer volume	1.5 mL (transferred from column 1 to column 2; volume depends on cutting interval)
Column oven temperature	25 °C
Detection	UV detector at 280 nm
Injection volume	1 mL

7 Evaluation

7.1 Method

Quantitation is performed using an external standardization procedure. Construct a new calibration curve with trinexapac-ethyl or trinexapac standard solutions with each set of analyses. Plot the peak area (trinexapac-ethyl for soil, water, or air) or peak height (trinexapac for soil or water) against the injected amount of trinexapac-ethyl or trinexapac. Before each set of measurements, check the HPLC system by injecting more than one standard solution.

7.2 Recoveries, limit of detection (LOD) and limit of quantitation (LOQ)

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7.2.1 Trinexapac-ethyl

Soil

With fortification levels between 0.04 and 0.2 mg kg^{-1}, recoveries from soil samples range from 79 to 104%. The average recovery is $90 \pm 6\%$ ($n = 19$). The LOQ is 0.02 mg kg^{-1}.

Water

With fortification levels between 0.10 and 10 µg L^{-1}, average recoveries from water samples range from 74 to 83%. The overall recovery is $77 \pm 13.2\%$ ($n = 25$). The LOQ is 0.10 µg L^{-1} and the LOD is 2.5 ng.

Air

With fortification levels between 20 and 200 ng L^{-1} based on 120 L of air, recoveries from the sorbent range from 75 to 83% with an average recovery of $77 \pm 3\%$ ($n = 6$). The LOQ is 20 ng L^{-1}.

7.2.2 Trinexapac

Soil

With fortification levels between 1 and 5 µg kg^{-1}, average recoveries from soil samples range from 83 to 92%. The overall recovery is $88 \pm 11\%$ ($n = 19$) and the LOQ

is 1 $\mu g\,kg^{-1}$. The LOD of the method was not formally assigned but can be set to 1 $\mu g\,kg^{-1}$.

Water

With fortification levels between 0.05 and 0.5 $\mu g\,L^{-1}$, average recoveries range from 92 to 96%. The overall recovery is $95 \pm 9\%$ ($n = 46$). The LOQ is 0.05 $\mu g\,L^{-1}$. The LOD of the method was not formally assigned but can be set to 0.05 $\mu g\,L^{-1}$.

7.3 Calculation of residues

The amount of trinexapac-ethyl or trinexapac residue, R ($ng\,g^{-1}$), in the sample is calculated by the following equations:

$$R\ (ng\,g^{-1}) = \frac{\text{analyte found (ng)}}{\text{sample injected (g)}} \times \frac{100}{\text{recovery (\%)}} \tag{1}$$

where R ($ng\,g^{-1}$) = residue found ($ng\,g^{-1}$), analyte found (ng) = analyte found (ng) determined with standard calibration solutions, sample injected (g) = sample injected (g) determined by Equation (2) or (2a), and recovery (%) = percentage recovery determined using Equations (3) and (4).

Soil

$$\text{Sample injected (g)} = W_s \times \frac{V_a}{V_e + E_v} \times \frac{V_i}{V_f} \tag{2}$$

Water and air

$$\text{Sample injected (g)} = W_s \times \frac{V_i}{V_f} \tag{2a}$$

where W_s = weight of the subsample (g) (air samples in L), V_a = volume of aliquot cleaned up (mL), V_e = volume of extraction solvent (mL), E_v = estimated volume of the extracted solute (mL), V_i = volume injected for determination (mL), and V_f = volume of final injection solution (mL)

$$\text{Recovery (\%)} = \frac{\text{analyte found in fortification} - \text{analyte found in control}}{\text{analyte fortified}} \times 100 \tag{3}$$

where

$$\text{Analyte found } (ng\,g^{-1}) = \frac{\text{analyte found (ng)}}{\text{sample injected (g)}} \tag{4}$$

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References

1. C.D.S. Tomlin (ed.), 'The Pesticide Manual,' British Crop Protection Council, Farnham, pp. 896–897 (2000).
2. E. Kerber, G. Leypoldt, and A. Seiler, 'CGA 163,935, A New Plant Growth Regulator for Small Grain Cereals, Rape and Turf,' Presented at the 1989 Brighton Conference – Weeds, Brighton, UK, November 20–23, 1989.
3. R.W. Daniels and S.K. Sugden, *Pestic. Sci.,* **47**, 363 (1996).

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Recent advances in analytical technology, immunoassay and other nonchromatographic methods

Regulatory considerations for environmental analytical methods for environmental fate and water quality impact assessments of agrochemicals

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1 Introduction

Accurate, precise and sensitive analytical methods are important to the collection of data needed for regulatory decisions about pesticide registration. This article describes the various components of analytical method development, validation and implementation that affect the collection of pesticide residue distribution data for regulatory assessment of environmental fate and water quality impacts. Included in this discussion are both the technical needs of analytical methods and the attributes of study design and sample collection needed to develop data that are useful for regulatory purposes.

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1.1 Regulatory perspectives

Pesticides are regulated in the United States under the framework of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), which ensures that pesticide registration decisions are based on a detailed assessment of the risks to humans and the environment that could arise from the product when used according to label directions. Environmental fate studies are needed to characterize the behavior of a pesticide when used as specified on the pesticide label and to estimate the level of environmental exposure to humans and nontarget organisms. Analytical methods developed for these studies must have adequate sensitivity to address regulatory questions and must be capable of being implemented (if the need arises) by Federal or State government agencies and independent laboratories conducting residue monitoring studies for research or enforcement once the outdoor usage of the product begins.

Currently, a battery of studies are required as a condition of registration primarily involving analysis of active ingredient and major metabolites in soil or water media.

Handbook of Residue Analytical Methods for Agrochemicals.
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Among the studies commonly required for registration of pesticides with outdoor uses are hydrolysis (generally in sterile, buffered solution), aqueous photolysis, soil photolysis, aerobic soil metabolism, anaerobic soil metabolism, aquatic aerobic or anaerobic soil metabolism, batch equilibrium soil adsorption/desorption, soil column leaching, and field dissipation (tracking dissipation of the pesticide in the field). Study requirements are described in more detail in 40 CFR, Part 158.[1] Specialized field-scale groundwater, runoff or larger scale groundwater and surface water monitoring may also be required to refine estimates of pesticide occurrence in water used in ecological risk or drinking water assessments.

The Food Quality Protection Act (FQPA), passed in 1996,[2] required assessment of the aggregate risk of pesticides from dietary exposure and other nonoccupational exposure routes such as drinking water and residential use. High-quality environmental fate and water residue data are needed to develop quantitative drinking water exposure assessments. This has significantly increased the need for methods with ultra-low sensitivity for some chemicals in field studies and for methods which are easily reproducible and transferable to a wide array of users. Although water monitoring had been required prior to FQPA to provide site-specific data on pesticide occurrence, the number of programs required increased as a way of providing data to refine exposure estimates. The focus of the programs also expanded to routinely include analysis of major degradates of registered pesticides in addition to the parent compound when these metabolites are included in the aggregate FQPA risk assessment.

In addition, the FQPA provisions necessitate that a cumulative risk assessment be performed on pesticides (and their degradation products) with a common mechanism of toxicity. There are several implications of this legislation for analytical method development. First, developing methods to determine, simultaneously, concentrations for all compounds with a common mechanism of toxicity at a specific site with time would better serve regulatory needs for cumulative exposure assessment than having to combine analysis of different compounds from samples representing different exposure periods at different sites. Second, because the effects of multiple compounds are added together in a cumulative risk assessment, there is a heightened need for accuracy of analytical methods at low concentrations, which may not be significant from an aggregate risk standpoint. US Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) recently completed the first major cumulative human health risk assessment required under FQPA for organophosphate pesticides with a common mechanism of toxicity. The cumulative exposure and risk assessment[3] describes additional data needs for assessing uncertainties on water exposure in more detail.

1.2 Scientific perspectives

The laboratory and field studies on environmental fate that are required to support pesticide registrations provide essential data for risk assessments both to describe the environmental fate for the conditions measured and as input to simulation models used to extrapolate the results to other environments. The design goals and analytical needs for these studies vary depending on where in the registration process they occur and the regulation question being addressed. Table 1 identifies several key design goals for studies used by USEPA in assessing a pesticide's environmental fate.

Table 1 Analytical parameters of pre-registration and post-registration pesticide environmental fate studies

Study type	Required pre- or post-registration?	Use of radiolabel	Identification of unknowns	Need for mass balance[a]	Importance of low detection limits	Importance of adjustable sampling frequency
Laboratory metabolism	Pre-	Yes	Yes	Yes	Low–medium	Medium
Adsorption/ desorption	Pre-	Yes	No	Yes	Low	NA[b]
Field dissipation	Pre-	No	No	No	Medium	Medium
Field leaching (prospective groundwater study)	Pre-/post-	No	No	No	High	Medium
Field runoff	Post-	No	No	No	High	High
Water monitoring surveys	Post-	No	No	No	High	Medium–high

[a] Mass balance cannot be strictly obtained in any open field study; however, in field-scale groundwater studies, accounting for as much of the applied material as possible in order to interpret the results is particularly important. With the pesticide diluted over a large mass of soil and groundwater, concentrations in some samples may be low and hard to detect, but the total mass leaching may be large.
[b] Not applicable.

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The need to understand the fate of pesticides in the environment has necessitated the development of analytical methods for the determination of residues in environmental media. Adoption of methods utilizing instrumentation such as gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), liquid chromatography/tandem mass spectrometry (LC/MS/MS), or enzyme-linked immunosorbent assay (ELISA) has allowed the detection of minute amounts of pesticides and their degradation products in environmental samples. Sample preparation techniques such as solid-phase extraction (SPE), accelerated solvent extraction (ASE), or solid-phase microextraction (SPME) have also been important in the development of more reliable and sensitive analytical methods.

1.3 Risk assessment perspective

Over the past decade, trends in the agrochemical industry have been towards developing application technologies to improve the placement of pesticides and to reduce off-target impacts on air, water, habitat and wildlife. At the same time, low application rate pesticides have been developed and increasingly used in agriculture. These low application rate pesticides produce low concentrations in the environment, which in some cases still need to be factored into risk assessments for regulatory decision-making because of their high toxicity to selected species. The development of analytical methods that can detect these compounds reliably at concentrations of environmental significance has been a real technical challenge.

As technology improves and more sophisticated analytical technologies become cost-effective for use in regulatory monitoring programs, questions arise about how to interpret the impacts of trace or low pesticide concentrations which occur in the environment. These questions are best answered by risk assessments that place the magnitude and extent of occurrence of the pesticide and its metabolites into a risk context, rather than using the toxicity of an individual pesticide to constrain the reporting of environmental measurements. The rationale for this is both the new paradigm of cumulative risk assessment and the need to address the fundamental regulatory question of what happens to a pesticide when it is applied to the environment. In addition, new toxicological endpoints are sometimes identified (for example, endocrine disruption) that underscore the importance of fully characterizing the entire distribution of concentrations that occurs in the environment even if the observed or predicted levels do not exceed currently identified levels of concern.

2 Acceptance criteria of environmental analytical methods for pesticide regulation

Collection of data on the environmental fate of pesticides and pesticide residues in the environment provides answers to specific regulatory questions about the mobility, persistence and transformation of pesticide residues under a given set of controlled conditions. Equally important is the use of those data to estimate the occurrence and concentrations of pesticide residues for a wider variety of conditions, for example, by using computer modeling or statistical techniques. The applicability of analytical methods, study design features and strength of the quality assurance (QA) and quality control (QC) program determine the precision and accuracy of the data generated and their usefulness both directly and indirectly as the basis for regulatory decisions. This section of the article describes the evaluation of and acceptance criteria for these analytical methods, Section 3 describes the important features of method development in relation to environmental data collection and Section 4 describes environmental sample analysis issues in the context of study design and sampling strategies.

2.1 Method submission and evaluation criteria

Pesticide registrants must supply EPA methods that are capable of producing accurate and precise data on the environmental fate and effects of their products (and submit study data obtained with such methodology). A high degree of reliability in risk assessments based upon modeling results (which in turn are based upon the registrant-supplied environmental fate study data) is only possible when the analytical data are of high quality. There are inevitably many uncertainties about the way in which the behavior of the chemical changes under different environmental conditions, and details regarding the use pattern, weather conditions and hydrogeological setting are often unknown. Limitations in these data often complicate the interpretation of analytical results. Furthermore, precise estimation of risk is difficult enough to obtain with reliable analytical data; precise estimation is impossible if the methods are not proven to be accurate and precise.

Methods submitted include single- and multi-analyte methods for parent compounds and for degradates of concern. Pesticide regulatory methods are needed for each type of environmental matrix; fate methods may be designed for soil, water, plant tissue, animal tissue or air, but are predominantly for soil and water. Analytical methods need to include a complete description of the procedure, materials and equipment in order to be completely reproducible. The methods should be practical and rapid and, to the extent possible while maintaining other quality objectives, inexpensive (often State and local regulatory agencies with few available resources need to utilize them).

Methods that are to be used to analyze for pesticide residues in environmental samples subsequent to registration must be able to provide fully confirmed detection and quantitation of residues above a minimum limit that is low enough to characterize the environmental fate of the pesticide. Methods that do not necessarily fully confirm the identity of the residues, such as most immunoassays and traditional gas chromatography (GC), high-performance liquid chromatography (HPLC) or even thin-layer chromatography (TLC) methods, can have a role in providing data for regulators, but the availability of confirmatory methods is still essential before the data can be used in regulatory decision-making. Mass spectrometry (MS) has been widely incorporated into methods for the unambiguous detection of a wide variety of pesticides in recent years, usually coupled with GC for less polar compounds and reversed-phase HPLC for more polar compounds. In recent years, MS technology has been incorporated into most environmental chemistry methods submitted to OPP.[4]

Registrants need to submit a complete description of performance and validation data for each method. Samples at each test level and each matrix (soil type, water source, etc.) should be submitted in adequate numbers with QC samples included in such a way as to permit the evaluation of method performance at each step in the procedure from storage to extraction to cleanup to analysis. The analytical data must support the limit of detection (LOD), the limit of quantitation (LOQ) and the accuracy and precision of the method. Method recovery data should include statistical representations (such as standard deviations and confidence limits) for each analyte at various levels of sample fortification. The relative standard deviation (RSD) of replicate recovery measurements should be within 20% at, or above, the LOQ for each sample. A particular method of calculation of the method detection limit (MDL) and LOQ is not mandated, but guidelines are provided in the Code of Federal Regulations which states that 'The (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero and is determined from analysis of a sample in a given matrix containing the analyte'.[5] Registrants must describe fully how they calculate the MDL and LOQ values. OPP may require revision of an analytical method if it has not been fully optimized or the chosen reporting standards based on the MDL and LOQ do not fully or accurately capture the information that can be obtained from the raw analytical results.

The OPP has three laboratories: the Environmental Chemistry Laboratory (ECL) in Bay St. Louis, MS, and the Analytical Chemistry Laboratory (ACL) and the Microbiology Laboratory at the Environmental Science Center, both at Fort Meade, MD. The ECL is heavily involved with method validation efforts. The ACL evaluates enforcement analytical methods for product chemistry to ensure that the ingredient statements on the label are accurate and evaluates residue analytical methods for

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detecting pesticide residues in food and feed to ensure that they are suitable for monitoring pesticide residues and enforcing legal residue limits (tolerances). The Microbiology Laboratory conducts product performance testing of disinfectants and public health-related antimicrobial products, evaluates new efficacy test methods for hospital disinfectants and serves as a reference laboratory on questions of test methodology and procedures.

2.2 Validation and availability of methods and standards

The availability of specific analytical methods for all analytes and matrices of concern at a reasonable cost must be a priority when designing an environmental fate or monitoring study. The EPA requires that methods used to support field data be submitted to the Agency for validation prior to acceptance (*Federal Register*: April 19, 1995; Notice OPP-00405; FRL-4943-5). If methods are unavailable for important degradates, or if unexpected stability problems arise in transport or storage before analysis, the usefulness of expensive field monitoring programs can be severely limited. Regulatory methods must be reproducible and widely adaptable to other agencies and laboratories desiring to monitor for the pesticide in some way once it is registered and being used. To ensure that this is the case, the OPP, through the ACL and ECL, conducts regular validation exercises of new analytical methods used in studies to support the registration of pesticides. Availability of analytical standards for pesticides and important degradates is assured through the EPA National Pesticide Standards Repository (Fort Meade, MD) operated by ACL. These pesticide analytical standards are provided only to United States Federal and State laboratories for the purposes of enforcement and compliance assurance, investigation and residue monitoring to support regulatory decisions.

The ECL evaluates analytical methods for detecting pesticide residues in the environment to ensure that the methods are suitable for monitoring pesticide residues in soil and water. State, tribal and federal laboratories may access an Index of Environmental Chemistry Methods for a list of available methods. The ECL also provides the State pesticide laboratories with technical and QA support and training in pesticide analytical chemistry.

The registrant may also be required to submit an independent laboratory validation (ILV) of their analytical methods. The registrant arranges for the ILV, unlike the validations conducted by ECL. The registrant can do the ILV within their own organization, but if they do, they must ensure that equipment and supplies used are different from those used in the laboratory where the method was originally developed. The personnel involved in the ILV must also be different and have no pre-existing experience with the method being validated. Whatever laboratory is chosen for the ILV, contact with the registrant or method developers must be limited to minor points of clarification in the procedure (such ambiguities can easily be resolved later by minor editing of the written procedure). QC procedures are important in determining whether the method performance is adequate. Up to three attempts may be made to get the method to perform adequately, with some additional contact with the registrant (if fully documented) allowed between each attempt. Additional details can be found in Ref. 6.

3 Specific method development issues

Studies conducted in the laboratory provide fundamental data on processes by which a pesticide is degraded and on its mobility. In combination with field observations, which integrate multiple processes, these data describe a pesticide's environmental fate. This section provides a discussion of several important specific analytical issues which should be considered in the design of environmental fate studies to ensure that the data generated address the needs of scientists and regulatory agencies for information on the environmental fate and environmental and ecological impacts of a pesticide to the fullest extent.

As probabilistic exposure and risk assessment methods are developed and become more frequently used for environmental fate and effects assessment, OPP increasingly needs distributions of environmental fate values rather than single point estimates, and quantitation of error and uncertainty in measurements. Probabilistic models currently being developed by the OPP require distributions of environmental fate and effects parameters either by measurement, extrapolation or a combination of the two. The models' predictions will allow regulators to base decisions on the likelihood and magnitude of exposure and effects for a range of conditions which vary both spatially and temporally, rather than in a specific environment under static conditions.[7] This increased need for basic data on environmental fate may increase data collection and drive development of less costly and more precise analytical methods.

3.1 Identification of unknowns/selection of analyte(s)

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Rigorous investigation of degradation pathways under a variety of environmental conditions is an essential component in fully characterizing a pesticide's environmental fate. Regulatory studies are therefore required which involve application of the pesticide to soil, water or soil–water mixtures and subsequent separation and characterization of hydrolytic, photolytic and metabolic products. Typically, radiolabeled pesticides are applied in these studies to facilitate this characterization and to achieve a material balance analysis (target 90–110% over the entire study[8]). The combination of laboratory investigations which identify key degradation products and field studies (which look for these compounds after application of the pesticide) often reveals that some degradates are much more pervasive environmental contaminants than the respective parents. For example, questions about aldicarb contamination of groundwater could not adequately be addressed by regulators until targeted monitoring programs were developed. These programs were, in a stepwise process, increasingly targeted to the relatively few crop use sites and soil types responsible for the majority of the impact on groundwater resources. As monitoring proceeded, specific methods had to be developed for the major metabolites of aldicarb which were critical to the risk assessments, especially since the sulfoxide and sulfone derivatives of aldicarb were known to be toxic and were proven to be responsible for the major amount of residues in groundwater.[9]

Typically, the EPA requires monitoring for specific pesticides and degradates. Degradates which need to be identified are major degradates [residues present at levels $\geq 10\,\mu g\,kg^{-1}$ (ppb) or 10% of applied], are mobile in the environment or have

identified toxicity. Initial laboratory and field studies done to support registrations should identify these compounds; however, additional methods may need to be developed for other compounds which contribute to the aggregate or cumulative exposure of the pesticide, e.g., formulation by-products, if the potential for exposure exists.

3.2 Detection limits/reporting limits

Methods must be extremely sensitive for environmental fate studies to support pesticide registration. Recent examples are methods developed for imidazolinone and sulfonylurea pesticide determinations.[10,11] One reason for this is that many of the newer classes of compounds that have been developed as pesticides are active on target organisms (and to a varying extent on nontarget organisms) at extremely low rates. Some modern pesticides are applied at rates of only a few grams per hectare, and concentrations of only 50–100 parts per trillion (nanograms per kilogram) in soil or water can still have effects on sensitive organisms.

EPA definitions for method sensitivity standards are provided in the Code of Federal Regulations (40 CFR, Part 136, Appendix B): 'The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero and is determined from analysis of a sample in a given matrix containing the analyte...' Pesticide registrants frequently will employ a Reporting Limit (RL) which can be used for all samples (i.e., the RL is uniformly applied to all samples whereas the MDL may be variable and therefore lower than the RL in some samples; matrix effects are not expected to be great enough in any specific sample to compromise the reliability of a quantitation at the RL). The RL is not defined in 40 CFR Part 136, but generally, EPA asks that if a RL is used, all detections below the RL but above the MDL still be reported separately.

Whenever samples from environmental fate studies are reported as nondetects (that is, not detectable within the capability of the method used) or even as nonquantified detects, the amount of information obtained on the pesticide fate is reduced, thus making more general conclusions on how the pesticide might behave much more difficult and uncertain. Also, fate and transport models cannot be validated using a dataset comprised of samples with nondetectable analytical results. Imagine for the data in Figure 1 that the method detection limit (MDL) for the pesticide was 100 units. This would reduce the number of sampling dates with quantitative data dramatically, as the median concentration was above 100 units (rather than the MDL of 5 units represented in the figure) only at one interval, some 2000+ days after application. Any information about the temporal pattern of occurrence would have been lost, and conclusions about breakthrough patterns of the pesticide would have been impossible. Also, with the high spatial variability of pesticide leaching (even in fields with vadose zone characteristics that superficially appear fairly homogeneous), any attempt at validation of a model using such sparse data would be highly suspect.

Even before a method is developed for detecting the presence of a pesticide or pesticides in the environment, the level of sensitivity in the method that will be needed for fate and monitoring studies to adequately portray the behavior of the analytes in the environment must be assessed. For example, in surface water monitoring programs,

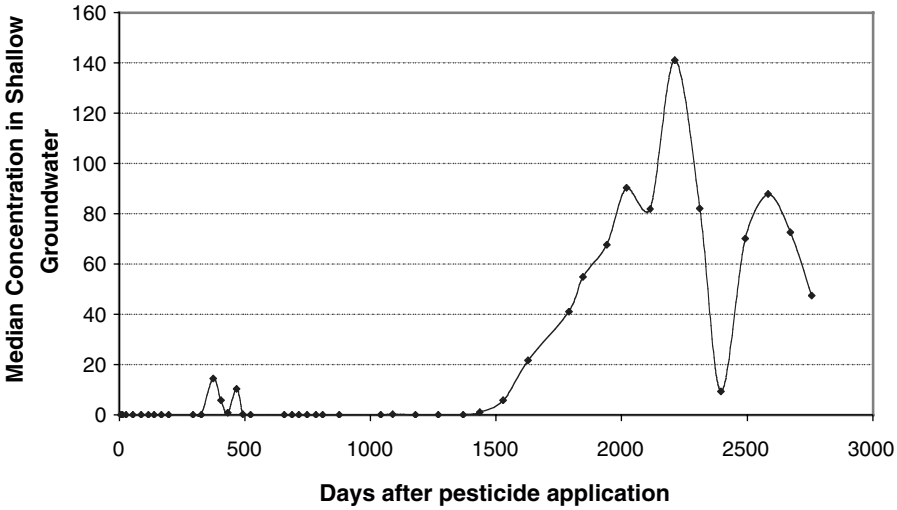


Figure 1 Example of pattern of appearance of a pesticide in shallow groundwater following a single application

many pesticides are present in a stream at low concentrations throughout much of the year and at high concentrations for relatively brief periods during a very few major runoff events during and after the application window (Figure 2 provides an example of this type of pattern of occurrence). This is a common type of pattern and may be due to residues first leaching in a field to groundwater and then taking perhaps months or years to reappear at the surface when groundwater discharges to form the base flow of a stream. Residues may flow from land to streams underground, but near the surface, when there is a restrictive layer in the soil or when tile drains are present, resulting in travel times that are shorter than for residues that reach deeper groundwater first,

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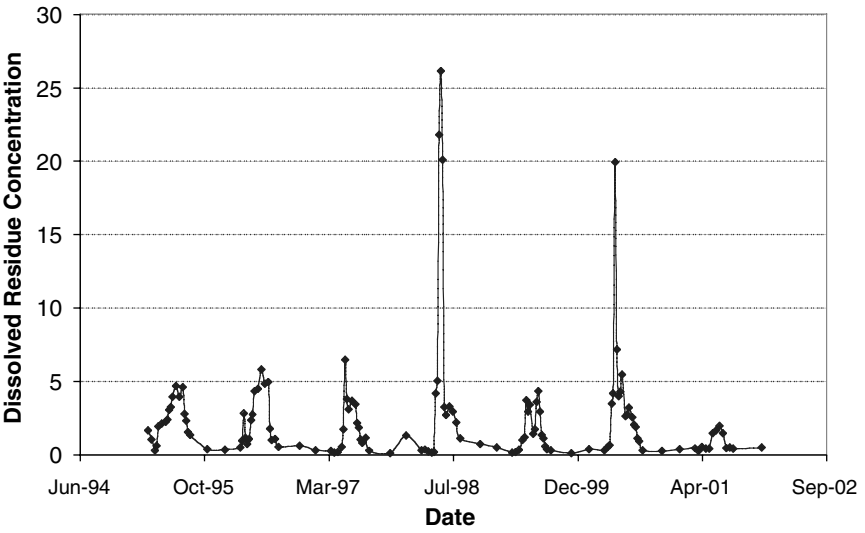


Figure 2 Pesticide stream concentrations, 1994–2002 (demonstration of temporal variability in concentrations)

but longer than with classic surface runoff. Typically, methods must be able to detect concentrations of a few nanograms per liter to adequately address the movement of pesticide into surface waters throughout the whole year. In pesticide leaching studies, high sensitivity in analytical methods is needed because residues may be spread over a high volume of the vadose zone and concentrations in soil pore water may be highly spatially variable. In subsequent model validation efforts, the low level detections in many samples are just as critical information as the high level detections that may occur in only a few samples.

Rather than simply ensuring that the analytical methods used have scientifically justifiable detection, reporting and quantitation limits, the level of sensitivity that will be needed for the type of fate or monitoring study being conducted must be determined first. In OPP's 'Guidance for Prospective Ground-Water Monitoring Studies' (scheduled to be released by the EPA by early 2003), the following guidelines are provided for these studies (which track leaching of a pesticide and a conservative tracer through the vadose zone and into groundwater in a single field):

> The MDL and practical quantitation limit (PQL) should be appropriate for the objectives of the analysis. MDL refers to the minimum concentration of the compound of interest that can be measured and reported with a specified confidence (99% probability) that the concentration is above zero. The registrants must provide or develop an analytical method for water for the parent pesticide and its degradates that has an MDL of 0.01% of the label application rate (calculated as the average concentration in the top six inches of soil), or $0.05\,\mu g\,L^{-1}$, whichever is lower. PQL refers to the lowest concentration at which the laboratory can confidently quantify the concentration of the compound of interest. The study authors must report all samples with concentrations above the MDL as detections, including those below the PQL in which the concentration cannot be quantified. In addition, the study authors must provide sample equations to demonstrate how the PQL was calculated.

While these objectives for method sensitivity may seem ambitious, experience has shown that data from such studies are much more usable for supporting fate and transport models (development and/or validation efforts) that may have to be used when more precise and geographically detailed probabilistic risk assessments become necessary.

3.3 Extraction efficiency/mass balance

The fundamental issue is to describe how much of the residue can be characterized accurately and whether an accounting of the applied mass of pesticide can be maintained throughout the course of the experiment. A series of environmental fate studies is required for pesticide registration in order to characterize the degradation pathways and formation and decline patterns of each major degradate. These studies are typically conducted in the laboratory under controlled conditions, applying radiolabeled pesticides to evaluate the extraction efficiency of various procedures. When standard extraction methods fail to release a significant amount of the applied radioactivity, more efficient and exhaustive extraction procedures are tried in a stepwise fashion

until most of the residues are extracted and can be characterized, hopefully without causing further degradation in the extraction process. The goal is to ensure that the degradates formed are adequately characterized (parent or degradates that might possibly be of toxicological concern) and to determine if residues are tightly adsorbed to soil or sediment or are likely to be bioavailable. Acceptable mass balance recoveries are defined as 70–110%.[8] If this level of mass balance is not achieved by the analytical methods used, the uncertainty in the results makes it difficult for regulators to draw accurate conclusions about mechanisms of degradation and potential exposure to pesticides from the necessarily limited number of environmental fate studies required for registration.

Sometimes, accounting for specific metabolic products takes on greater importance than might be thought from the amount of the metabolite observed in an environmental fate laboratory study. For some pesticides, degradation products are formed that are significantly more mobile in soil and more persistent in soil and water than the parent compound from which they are derived. These compounds can be the most significant contaminants of groundwater or surface water arising from the field use of the pesticide even when they are not quantitatively the most important products formed in laboratory studies. Acetanilide herbicide degradates (ethanesulfonic acid and oxanilic acid derivatives of parent compounds such as alachlor, acetochlor and metolachlor) are examples of compounds that are more prevalent and are found at higher concentrations than the respective parent compounds in groundwater and surface water. Even so, in laboratory studies submitted to support the registration of these products, the amount of these degradates detected rarely amounted to more than 5–10% of the applied parent compound. Therefore, a full accounting of the fate of the parent compound over time along with positive identification of all significant metabolites must be obtained in these studies.

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3.4 Matrix effects

Large-scale water monitoring studies may be needed to evaluate the impacts of pesticides in a number of locations post-registration. Samples may have to be taken from water with a variety of characteristics that can influence the stability and extractibility of the pesticides after sample collection. The physical properties of the pesticide are also important, as compounds vary in their degree of partitioning into solution. Some insecticides that tend to be adsorbed rapidly and tightly on organic and other colloidal material in suspension in the water are nonetheless of toxicological concern because of the potential acute effects to sensitive organisms. Accurate determination of such lipophilic pesticides is complicated by the fact that recoveries may vary widely between different types of water samples.

Processes that significantly change the acid–base characteristics or redox properties of the water may potentially produce transformation products. These transformation products may be significant in assessments of pesticide exposure from drinking water.[12] For example, data from an EPA–United States Geological Survey (USGS) pilot reservoir monitoring project, which collected paired pre- and post-treatment samples, indicate that water treatment processes have an impact on the recovery of organophosphates in treated water when compared with fortified raw water samples,

presumably due to oxidation by residual chlorine.[13] Hence, the analysis of this water matrix (treated drinking water) poses its own set of analytical issues.

4 Specific environmental sample analysis issues

Decisions made in the design of field study data collection directly and indirectly affect analytical method development. Each sampling matrix will require specific procedures, and methods need to be developed with a view to the nature and scope of field monitoring programs that are or may be required.

4.1 Identification of target population in monitoring programs

The selection of the target population ('population' here is used in the statistical sense of the domain from which the sampling occurs) is dependent on the regulatory question to be addressed. Among the target populations that have been the focus of water monitoring studies are:

- actual ('finished') drinking water
- 'raw' or pre-treatment drinking source water
- shallow groundwater at the edge of agricultural fields
- actual drinking water wells
- primary and secondary streams in agricultural areas
- primary and secondary streams in urban areas
- major rivers
- dissolved concentrations
- total (dissolved + suspended) concentrations
- small reservoirs with slow flow-through.

Each of these is an example of the types of water that have been targeted in pesticide monitoring studies. Finished water studies provide direct measurement of exposure but do not provide much information on the effect of pesticide use patterns, weather and treatment processes on drinking water exposure. Raw water studies do not provide information on the impact of treatment processes on exposure, but such insight could be gained by additionally analyzing finished water along with collection of data on treatment processes used by each facility. Each of the other target populations likewise is especially suited to answer different sorts of questions: small stream studies may be chosen for ecological effects, large rivers may be chosen for looking at exposures of large human populations, and small reservoirs may be chosen to look at upper-bound drinking water concentrations.

The emphasis that the FQPA placed on the assessment of pesticide residues in drinking water, for example, led to the collection and analysis of data on the effects of drinking water treatment processes on pesticide residues. These data were presented to the FIFRA Science Advisory Board[14] to highlight the variability in the effects of treatment on different kinds of pesticides and the products formed and the variability of treatment processes employed at different locations and at different collection time intervals at an individual location. These complexities led to the current proposal

that, if data are to be collected for drinking water assessment and may be used also to develop mitigation measures based on label requirements and agricultural practices, a mixture of pre- and post-treatment water samples should be collected and analyzed at each monitoring location. The pre-treatment samples will contain detectable residues from pesticide use in the upstream watershed, while post-treatment samples will represent the combined impact of water treatment processes (coagulation, softening, flocculation, chlorination, etc.) on reducing these pesticide residues.

4.2 Sample collection strategy: study design

When a monitoring study focuses on one or two pesticides, developing a sampling strategy (assuming that the target population is first correctly identified) that will efficiently measure the impact on water quality is relatively easy. Multi-compound monitoring programs, such as the EPA–USGS Pilot Reservoir Monitoring Program,[13] and proposed designs for a national survey include a variety of strategies to select target pesticides and sampling schemes. The cost constraints behind large surveys and any monitoring which is large in scope play a significant role in design decisions: there is an intimate link between site selection, chemical selection, sampling frequency and the cost constraints of these studies. One way to maximize the information obtained and to minimize analytical costs is to use multi-analyte methods, which detect and quantitate a large number of pesticides in one analysis. A downside of multi-analyte methods is that the data obtained may not be equally meaningful for all analytes. For example, if a particular pesticide is not used particularly near most of the sampling locations, a multi-analyte method will provide data on the impact of the pesticide on water quality in areas where the pesticide is not used. One cannot conclude that these survey results for that pesticide represent the impact on water quality in areas where that particular pesticide is actually used extensively and where the pesticide is most prone to runoff to streams or leach to groundwater.[13,15]

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4.2.1 Spatial scope of study

When monitoring is required to characterize the impact of a specific pesticide on water quality, the initial scope of the study chosen is dependent on an analysis of the pesticide use area. When the study is large-scale and involves a large number of pesticides, a great amount of information is needed on use patterns for all of the analytes in recharge zones (for groundwater sampling) or watersheds (for surface water sampling) associated with each sample.[14–16] Furthermore, this information may be needed for a considerable period of time, as in the common case where pesticides sometimes do not fully impact groundwater until several years or more after application (Figure 1). When a relatively small number of pesticides are the target analytes in a study, overlaying of maps of use intensity and factors associated with either groundwater or surface water vulnerability to contamination can help provide focus on a subset of geographic locations for monitoring. Other factors which can be used to further narrow the geographic scope of a study are watershed delineation, drinking water intake locations (either public or private), specific pesticide uses, specific agronomic practices and knowledge about the environmental fate properties of a pesticide (e.g., pH-dependent hydrolysis),

which can be expected to reduce or increase exposure in some geographic locations. As geographic information systems (GIS) tools advance and spatial datasets become more available, such analyses should become increasingly feasible. Multi-compound monitoring studies will have a larger scope, and cost considerations again play a larger role. Multi-analyte methods or immunoassay screens when used correctly should result in very few false negatives (failure to detect the pesticide when it is in fact present at levels equivalent to the stated sensitivity of the method), and a manageable rate of false positives (which can be dealt with by reanalyzing the relevant samples with confirmatory methods) can be quite useful to control analytical costs in large-scale studies.

Lessons have also been learned about the importance of carefully considering the scope of the study in large-scale monitoring surveys to support the registrations of specific pesticides, as in studies for acetochlor,[17] alachlor,[18] aldicarb[9] (discussed previously) and atrazine.[19] In the acetochlor surface water monitoring study on drinking water facilities, the lack of analysis of raw drinking water at many sites meant that the effectiveness of various drinking water treatment processes at these sites could not be evaluated. In the alachlor study, mixing of other herbicides as analytes provided interesting comparative information; however, since the samples were chosen on the basis of alachlor use alone, one could not evaluate fully how representative the study results were of the impact of use of these other herbicides. In the atrazine groundwater studies, different criteria were used to select wells for monitoring in every State, providing a greater ability to focus on groundwater at high risk of contamination but making comparision of results from State to State of difficult.

4.2.2 Sampling frequency

As shown in Figure 2, pesticides flowing in surface waters tend to occur in pulses. The patterns of appearance in surface water are closely related to seasonal application trends, weather (especially, precipitation patterns) and the flow patterns of the water body. The pulses can last from hours to days to weeks to months, depending on the weather, type of water body and the pesticide properties and use patterns (both timing and method of application). Martin[20] provides a detailed assessment on the seasonal occurrence of most pesticides in surface water as related to the timing of pesticide applications, rainfall or irrigation and the size of the watershed. Decreasing uncertainty in assessments of pesticide occurrence is directly related to the number and frequency of samples collected; this is an especially important issue for pesticides which may not be persistent in most natural waters but are known to exhibit adverse effects on some organisms even after exposures of very short duration. The optimum frequency of sampling depends on local use and weather patterns for each pesticide and varies among pesticides and among geographical regions.

4.2.3 Study duration

Climatic fluctuations, long-term usage trends and agronomic practices can dramatically affect the characteristics of the monitoring data obtained from a field study, and this in turn affects the degree to which the study can be utilized to generalize about the environmental impacts of use of the pesticide. Surface water programs should be multi-year studies if one intends to address adequately the variability of pesticide

loadings to these waters that occur in a range of weather patterns (and also with varying use and management practices from year to year).[21] Some pesticides take several years or more to enter groundwater or can take years after application to reach streams and rivers by subsurface flow (in the example illustrated in Figure 2, pesticide residues took 5–8 years to leach to groundwater about 15 ft below the surface). The implication of sampling frequency and study duration on method development is that the laboratory procedures and setup must be able to accommodate the scope of the study and, perhaps, increased sample burden for the laboratory during periods when sampling must be more intense or when quick turnaround of samples is needed in order to optimize future scheduling of sample collection.

4.2.4 Ancillary data collection

Several issues come into play in interpreting ancillary data, many of which revolve around the techniques used for sample collection, storage, analysis and reporting of monitoring data. Monitoring data, if they are to have scientific and regulatory value, must not be generated in a vacuum. The analytical data generated are useful for regulatory purposes only when associated with information on the use of the pesticide (geographically and temporally specific), local hydrogeology, topography, soil characteristics, land use (including soil surface features, ground cover and management features) and the hydrology of the water body being sampled.[22,23] For example, interpretation of the spikes in concentrations that often occur in stream water (Figure 2) is only possible by knowing with some precision the timing, magnitude and spatial distribution of pesticide applications within the watershed, the precipitation patterns, the land surface features and the physicochemical properties of the pesticide. For some relatively nonpersistent pesticides, ecological effects may be significant because major environmental effects can occur from very short-term exposure of organisms to toxicologically significant levels of the chemical. The ancillary data collected not only facilitate the interpretation of the monitoring results but also provide a reality check, answering questions such as 'Is it reasonable to expect a sharp spike in the concentration of this pesticide at this particular time?'.

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4.3 Effect of inert ingredients

In addition to toxicity per se of registered active ingredients in pesticide formulations, so-called inert ingredients may have some environmental impact either through toxicity of their own or through their impact on the environmental fate of the active ingredient. As defined by Federal law (FIFRA), an active ingredient is one that prevents, destroys, repels or mitigates a pest, or is a plant regulator, defoliant, desiccant or nitrogen stabilizer. An inert ingredient is simply any ingredient in the product that is not intended to affect a target pest. For example, isopropyl alcohol may be an active ingredient and antimicrobial pesticide in some products; however, in other products, isopropyl alcohol is used as a solvent and may be considered an inert ingredient. Inert ingredients are not necessarily nontoxic, and current label requirements list these substances as 'other ingredients' to preclude propagation of the erroneous assumption sometimes made that all inerts are nontoxic. Types of inert ingredients

included in pesticide formulations are stickers (to increase the time during which the pesticide remains on the plant or other surface), solvents, surfactants (to modify surface characteristics such as reducing the surface tension of water) and carriers such as clay (upon which the active ingredient is coated).

4.4 Field quality control issues

Monitoring programs may be required for pesticides that are registered in order to refine exposure estimates made using laboratory-derived data and screening level computer simulation models. The monitoring studies should be designed so that data are useful in developing and validating more advanced models used to estimate exposure and in evaluating the magnitude of risk reduction that will result from mitigation alternatives. Without careful attention to sampling design and collection of the appropriate data for interpretation of monitoring results, the analytical data may not be useful for regulatory purposes.

4.4.1 Stability in transit and storage

In many cases, there is difficulty in preserving residues in samples after collection and prior to pesticide analysis which coincides with a rapid further degradation and mineralization of the pesticide residues under most environmental conditions. Storage stability studies and studies on the reactivity of sample collection equipment in addition to field quality assurance procedures can help address some of these questions. Concerns are accentuated for compounds that have short half-lives in the environment but still have high acute toxicity.

4.4.2 Sample contamination

As more sensitive analytical methods for pesticides are developed, greater care must be taken to avoid sample contamination and misidentification of residues. For example, in pesticide leaching or field dissipation studies, small amounts of surface soil coming in contact with soil core or soil pore water samples taken from further below the ground surface can sometimes lead to wildly inaccurate analytical results. This is probably the cause of isolated, high-level detections of pesticides in the lower part of the vadose zone or in groundwater in samples taken soon after application when other data (weather, soil permeability determinations and other pesticide or tracer analytical results) imply that such results are highly improbable.

The spatial and temporal scope of the study determines the quantity of samples that require processing and storage and can put great pressures on laboratory capacity, potentially increasing the risk of compromising sample integrity and affecting the study quality.

4.4.3 Quality control applied to large-scale monitoring studies

Many sources of uncertainty must be taken into account in interpreting water quality data collected in the field. Probably the single program that has most prominently

addressed these issues is the USGS National Water Quality Assessment Program (NAWQA). To date, NAWQA is the largest, most extensive in temporal coverage and arguably the most sophisticated national-scale program to describe the status and trends in water quality with regard to a large number of pesticides and degradates. The USGS devoted substantial resources to laboratory and field method development and documentation of QA and QC. An outgrowth of the NAWQA study has been some excellent examples of how QC information can be used in the analysis and interpretation of environmental data. Field replicate samples were used to assess variability and the rate of false positive and false negative errors, and Martin[24] concluded from these data that the major source of variability (inconsistency) in the methods was variability in the analytical method and water matrix interferences. A low rate of false positives was ascertained from a low rate of 'detections' from the field blanks, implying that sample contamination was not a major source of inconsistency in the performance of the methods.

4.4.4 Dynamic evaluation of method performance

Analytical method modifications during the course of a study must also be documented and can provide an explanation of anomalous results. Particularly in large-scale monitoring surveys, samples may come in matrices that vary considerably in their physicochemical properties, affecting the behavior of the analytes in sample storage and the extractability of the analytes from the matrix. These issues can be assessed with the use of instrument blanks, field replicates, field fortification samples and field fortification replicates from representative sample sites. Instrument blanks can show if sampling equipment is responsible for sample contamination. Field replicates, field fortifications and field fortification replicate samples illustrate variability in concentrations in the field, shipping and storage losses and the precision and accuracy of sampling and laboratory analyses. Additionally, internal standard or surrogate compounds can be added to samples prior to extraction to assess losses in storage, the efficiency of the extraction technique and potential matrix effects.

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5 Conclusions: regulatory context

The EPA uses environmental fate and water quality data to evaluate exposure to and risk from pesticides in a tiered process. Models and monitoring data are used to determine the upper-bound exposure levels to pesticides and determine whether significant exposure and risk might occur with registered uses. Additional, geographically specific and probabilistic assessment is performed if the initial tiers indicate potential risks. Failure to conclude that there is no probability of adverse effects at the lower tiers of this assessment means that a much more time-consuming and costly risk assessment must be conducted for a pesticide. Also, with the regulatory mandate from FQPA for assessing cumulative risk, this risk assessment may have to be conducted simultaneously for multiple pesticides with different use patterns.

Ideally, to support a higher tier assessment that accurately portrays the risks to individuals depending on where they live and who they are, one would have monitoring and pesticide usage data from across the nation for multiple years in such exquisite

detail that a watershed by watershed and groundwater recharge area by groundwater recharge area acute and chronic exposure assessment could be performed directly from the monitoring data, leading to a precise calculation of risk for every individual. Such data are not available, and while the quality and quantity of such data are likely to increase over time, the data are unlikely ever to be sufficient to be used alone for a comprehensive pesticide risk assessment. What is required, therefore, is that a combination of monitoring and modeling (that is continually reassessed and validated with quality monitoring studies) be used for risk assessments. Future efforts at improvement in assessing exposure must address both the improvement of monitoring study design and model ability to characterize exposure under a variety of conditions.

In the past, risk assessments have had to be made with vastly inferior monitoring data and/or more primitive exposure models. Many 'false negative' conclusions were drawn because monitoring data were not collected at the right time, with adequate frequency and in the right place such that the impact of the use of a particular pesticide could be accurately assessed. Analytical methods were insufficiently sensitive to detect the pesticide in many cases. Also, since in most early monitoring studies insufficient pesticide usage, weather and other data were collected to assess when pesticide concentrations were most likely to be high, sampling was not done at the right time to detect the pesticide anyway. Pesticide degradates which have now been shown to be more prominent water contaminants were not even looked for. The scientific literature of the 1970s and 1980s is replete with research articles and monitoring study reports concluding that pesticides were not prevalent in groundwater or surface water that were later shown to be more common contaminants. Compounds such as prometon, tetrachloroterephthalate acid (degradate of DCPA), aldicarb (sulfone and sulfoxide degradates), metolachlor (parent and degradates), alachlor (ethanesulfonic acid and oxanilic acid degradates) and dibromochloropropane (DBCP) are all examples of compounds where the early literature revealed few cases of groundwater or surface water contamination, but later studies have shown much more widespread contamination in areas with significant use. Quality analytical methods that are reproducible and widely available to those trying to assess exposure from pesticides are essential to ensure that such oversights are not repeated in the future.

The views expressed in this article are entirely the authors', and do not represent or reflect the policy of the Environmental Protection Agency or any other entity of the United States government.

References

1. Code of Federal Regulations, Protection of the Environment; Title 40, Parts 150–189. Office of the Federal Register, National Archives and Records Administration, Washington, DC (1990).
2. Food Quality Protection Act, Public Law 104–170, August 3, 1996, 110 STAT 1489–1538. USEPA Office of Pesticide Programs, Washington, DC (1996). Also available on the World Wide Web: **http://www.epa.gov/oppfead1/fqpa/backgrnd.htm**, accessed August 2002.

3. Organophosphate Pesticides: Revised OP Cumulative Risk Assessment USEPA, Washington, DC (2002). Also available on the World Wide Web: **http://www.epa.gov/pesticides/cumulative/rra-op/**, accessed September 2002.
4. S.J. Lehotary, 'Multi-class, multi-residue analysis of pesticide, strategies for,' in "Encyclopedia of Analytical Chemistry: Instrumentation and Applications," ed. R.A. Meyers, Wiley, Chichester, pp. 6344–6384 (2000).
5. Definition and Procedure for the Determination of the Method Detection Limit – Revision 1.11, Code of Federal Regulations, 40. Protection of Environment, CFR Part 136, Appendix B, pp. 565–567. USEPA, Washington, DC (1992). Also available on the World Wide Web: **http://www.access.gpo.gov/nara/cfr/cfrhtml_00/Title_40/40cfr136_main_00.html**, accessed September 2002.
6. Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods "Public Draft," EPA 712-C-96-348. USEPA, Washington, DC (1996).
7. A Case Study: Advancing Ecological Risk Assessment Methods in the EPA, Office of Pesticide Programs, Final Report from FIFRA Scientific Advisory Panel. USEPA, Washington, DC (2001). Also available on the World Wide Web: **http://www.epa.gov/scipoly/sap/2001/index.htm**, accessed September 2002.
8. Pesticide Reregistration Rejection Rate Analysis. Environmental Fate. EPA 738-R-93-010. USEPA, Washington, DC (1993).
9. R.L. Jones and T.L. Estes, *J. Contam. Hydrol.,* **10**, 107 (1995).
10. E.T. Furlong, M.R. Burkhardt, P.M. Gates, S.L. Werner, and W.A. Battaglin, 'Routine determination of sulfonylurea, imidazolinone, and sulfonamide herbicides at nanogram-per-liter concentrations by solid-phase extraction and liquid chromatography/mass spectrometry,' in "US Geological Survey Toxic Substances Hydrology Program – Proceedings of the Technical Meeting, Charleston, South Carolina, March 8–12, 1999," eds. D.W. Morganwalp and H.T. Buxton, Vol. 2 of 3. Contamination of Hydrologic Systems and Related Ecosystems: US Geological Survey Water-Resources Investigations Report 99-4018B. US Geological Survey, Washington, DC, pp. 275–288 (1999).
11. Plan of Study to Determine the Occurrence of Sulfonylurea, Sulfonamide, and Imidazolinone Herbicides in Surface and Ground-Water of the Midwestern United States. US Geological Survey, Washington, DC (1998). Also available on the World Wide Web: **http://webserver.cr.usgs.gov/midconherb/html/workplan98.html**, accessed September 2002.
12. 'Preliminary Literature Review of the Impacts of Water Treatment on Pesticide Removal and Transformations in Drinking Water,' in "Progress Report on Estimating Pesticide Concentrations in Drinking Water and Assessing Water Treatment Effects on Pesticide Removal and Transformation: a Consultation," Session VI. USEPA, Washington, DC (2000). Also available on the World Wide Web: **http://www.epa.gov/scipoly/sap/2000/index.htm**, accessed September 2002.
13. Pesticides in Selected Water-supply Reservoirs and Finished Drinking Water, 1999–2000: Summary of Results from a Pilot Monitoring Program. Open-File Report 01-456. US Geological Survey, Washington, DC (2001). Also available on the World Wide Web: **http://md.water.usgs.gov/nawqa/**, accessed September 2002.
14. Consultation – June 6–9, 2000: National Drinking Water Survey Design for Assessing Chronic Exposure. USEPA, Washington, DC (2000). Also available on the World Wide Web: **http://www.epa.gov/scipoly/sap/2000/ index.htm**, accessed August 2002.
15. J.E. Barbash, G.P. Thelin, D.W. Kolpin, and R.J. Gilliom, Distribution of Major Herbicides in Ground Water of the United States. US Geological Survey Water-Resources Investigations Report 98-4245. US Geological Survey, Sacramento, CA (1999).
16. US Department of the Interior, Pesticides in Surface Waters, Current Understanding of Distribution and Major Influences. Fact Sheet 039-97. US Geological Survey, Washington, DC (1997).
17. USEPA, Acetochlor Web Page: **http://www.epa.gov/oppefed1/aceto/**, accessed August 2002.
18. L.R. Holden, J.A. Graham, R.W. Whitmore, W.J. Alexander, R.W. Pratt, S.K. Liddle, and L.L. Piper, *Environ. Sci. Technol.,* **26**, 935 (1992).
19. L.G. Ballentine, J.E. McFarland, and D.S. Hackett (eds), 'Triazine Herbicides: Risk Assessment.' American Chemical Society Symposium Series, No. 683. American Chemical Society, Washington, DC (1998).

DropBooks

20. J.D. Martin, Quality of Pesticide Data for Environmental Water Samples Collected for the National Water-Quality Assessment Program, 1992–96, and Examples of the Use of Quality-control Information in Water-quality Assessments. US Geological Survey, Washington, DC (1999). Also available on the World Wide Web: **http://ca.water.usgs.gov/pnsp/rep/qcsummary/**, accessed August 2002.
21. ILSI, A Framework for Estimating Pesticide Concentrations in Drinking Water for Aggregate Exposure Assessments, Workshop Report 5/19/99. International Life Sciences Institute, Washington, DC (1999).
22. S.J. Larson, P.D. Capel, and M.S. Majewski, 'Pesticides in Surface Waters: Distribution Trends and Governing Factors.' Ann Arbor Science Press, Chelsea, MI (1997).
23. J.E. Barbash and E.A. Resek, 'Pesticides in Ground Water, Distribution, Trends, and Governing Factors.' Ann Arbor Science Press, Chelsea, MI (1996).
24. J.D. Martin, Variability of Pesticide Detections and Concentrations in Field Replicate Water Samples Collected for the National Water-quality Assessment Program, 1992–97, USGS WRI 01-4178. US Geological Survey, Washington, DC (2001). Also available on the World Wide Web: **http://water.wr.usgs.gov/pnsp/**, accessed August 2002.

Immunoassay, biosensors and other nonchromatographic methods

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1 Introduction

Nonchromatographic methods for residue detection consist of a wide variety of techniques. For illustrative purposes these may be divided into 'biological'- and 'physical'-based methods, based on whether or not biological reagents are involved. Biological techniques include immunoassays, biosensors, bioassays, enzyme assays and polymerase chain reaction (PCR). Among the physical techniques that fit this category are spectrophotometry and voltammetry. The focuses of this article are the 'biological' techniques, in particular immunoassays and PCR, with a brief introduction to biosensors.

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2 Immunoassay for pesticides

The concept of immunoassay was first described in 1945 when Landsteiner suggested that antibodies could bind selectively to small molecules (haptens) when they were conjugated to a larger carrier molecule.[1] This hapten-specific concept was explored by Yalow and Berson in the late 1950s, and resulted in an immunoassay that was applied to insulin monitoring in humans.[2,3] This pioneering work set the stage for the rapid advancement of immunochemical methods for clinical use.

The first application of immunologically based technology to pesticides was not reported until 1970, when Centeno and Johnson developed antibodies that selectively bound malathion.[4] A few years later, radioimmunoassays were developed for aldrin and dieldrin[5] and for parathion.[6] In 1972, Engvall and Perlman introduced the use of enzymes as labels for immunoassay and launched the term enzyme-linked

Handbook of Residue Analytical Methods for Agrochemicals.
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immunosorbent assay (ELISA).[7] In 1980, Hammock and Mumma[8] described the potential for ELISA for agrochemicals and environmental pollutants. Since then, the use of immunoassay for pesticide analysis has increased dramatically. Immunoassay technology has become a primary analytical method for the detection of products containing genetically modified organisms (GMOs).

The advantages of immunoassay technology relative to other analytical techniques have been discussed in several reviews,[8–12] and include the following:

- low detection limits
- high analyte selectivity
- high throughput of samples
- reduced sample preparation
- versatility for target analytes
- cost effectiveness for large numbers of samples
- adaptability to field use.

As is the case with every analytical method, immunoassay technology has limitations, including:

- interferences from sample matrices
- cross reactivity to structural analogs of the target analyte
- poor suitability for some multi-analyte applications
- low availability of reagents
- longer assay development time than some classical analytical methods
- a large number of anticipated samples required to justify the development of a new assay for an analyte of interest.

The immunoassay is clearly not the best analytical method for all analytes in all situations. For example, gas–liquid chromatography (GLC) remains the method of choice for the analysis of volatile compounds. However, immunoassay technology is important for the analyst because it complements the classical methods, thus providing a confirmatory method for many compounds and the only reasonable analytical choice for others.[13] Most immunoassays can be used to obtain quantitative results with similar or greater sensitivity, accuracy and precision than other analytical methods. They are generally applicable to the analysis of small molecules, including pharmaceuticals and pesticides, identification of pest and beneficial species, characterization of crop quality, detection of GMOs, product stewardship, detection of disease and even monitoring for bioterrorism.

2.1 Principles of immunoassays

Immunoassays are based on the reaction of an analyte or antigen (Ag) with a selective antibody (Ab) to give a product (Ag–Ab) that can be measured. The reactants are in a state of equilibrium that is characterized by the law of mass action (Figure 1).

Several types of labels have been used in immunoassays, including radioactivity, enzymes, fluorescence, luminescence and phosphorescence. Each of these labels has advantages, but the most common label for clinical and environmental analysis is the use of enzymes and colorimetric substrates.

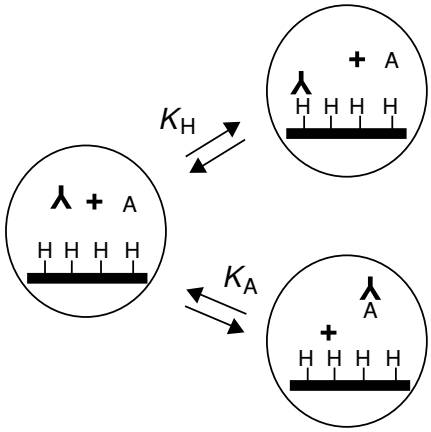


Figure 1 Schematic of the quasi-equilibria using heterologous haptens in coating antigen immunoassay formats. K_A represents the equilibrium constant for binding of antibody (**Y**) to target analyte (**A**). K_H is the equilibrium constant for the binding of antibody to hapten–protein conjugate (**H**–) immobilized on a solid phase

Enzyme immunoassays can be divided into two general categories: homogeneous and heterogeneous immunoassays. Heterogeneous immunoassays require the separation of bound and unbound reagents (antibody or antigen) during the assay. This separation is readily accomplished by washing the solid phase (such as test-tubes or microtiter plate wells) with a buffer system. Homogeneous immunoassays do not require a separation and washing step, but the enzyme label must function within the sample matrix. As a result, assay interference caused by the matrix may be problematic for samples of environmental origins (i.e., soil, water, etc.). For samples of clinical origin (human or veterinary applications), high target analyte concentrations and relatively consistent matrices are often present. Thus for clinical or field applications, the homogeneous immunoassay format is popular, whereas the heterogeneous format predominates for environmental matrices.

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2.2 *Immunoassay formats*

The microplate ELISA test is conducted in standard 96-well microplates. A microplate consists of a 12 × 8 grid of wells for test solutions. The three most widely used ELISA formats are immobilized antigen competitive immunoassay, immobilized antibody competitive immunoassay and sandwich immunoassay.[14,15]

The following is a generic description of the immobilized antigen ELISA (Figure 2), commonly termed indirect competitive immunoassay, on a microtiter plate.

Preparation of microtiter plates. A constant amount of the coating antigen is bound to the surface of polystyrene microtiter plate wells by passive adsorption. After a predetermined incubation time, the plate is washed to remove unbound coating antigen.

Competitive inhibition. A constant amount of anti-analyte antibody (primary antibody) and a series of solutions containing increasing amounts of analyte are added to the prepared microtiter plate wells. During incubation, the free analyte and bound

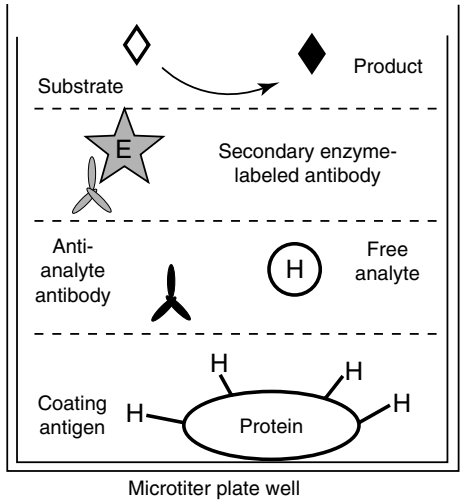


Figure 2 Immobilized antigen ELISA format. Antigen is immobilized to a solid phase by passive adsorption. Following removal of unbound antigen, analyte (free **H**) and antigen (**H**–protein) compete for a fixed number of primary antibody (**Y**) binding sites. Unbound materials are removed (dotted line). Secondary antibody–enzyme conjugate (**Y**–**E**) is added to bind to primary antibody followed by another wash step. Substrate (◇) for the enzyme is added to detect the bound enzyme. The amount of colored product (◆) detected is inversely proportional to the amount of analyte present

coating antigen compete for binding to antibodies in the mixture. Unbound reagents are washed out.

Secondary antibody and determination. A secondary antibody labeled with an enzyme is added which binds to the primary antibody that is bound to the coating antigen. If the primary antibody were produced in a rabbit, an appropriate secondary antibody would be goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) (or another enzyme label). Excess secondary antibody is washed away. An appropriate substrate solution is added that will produce a colored or fluorescent product after enzymatic conversion. The amount of enzyme product formed is directly proportional to the amount of first antibody bound to the coating antigen on the plate and is inversely proportional to the amount of analyte in the standards.

Another commonly used ELISA format is the immobilized antibody assay or direct competitive assay (Figure 3). The primary anti-analyte antibody is immobilized on the solid phase and the analyte competes with a known amount of enzyme-labeled hapten for binding sites on the immobilized antibody. First, the anti-analyte antibody is adsorbed on the microtiter plate wells. In the competition step, the analyte and enzyme-labeled hapten are added to microtiter plate wells and unbound materials are subsequently washed out. The enzyme substrate is then added for color production. Similarly to indirect competitive immunoassay, absorption is inversely proportional to the concentration of analyte. The direct competitive ELISA format is commonly used in commercial immunoassay test kits.

Sandwich ELISAs (Figure 4) are the most common type of immunoassay used for the detection of proteins. A capture antibody is immobilized on the wells of a microplate. The solution containing the analyte is introduced and antibody–analyte

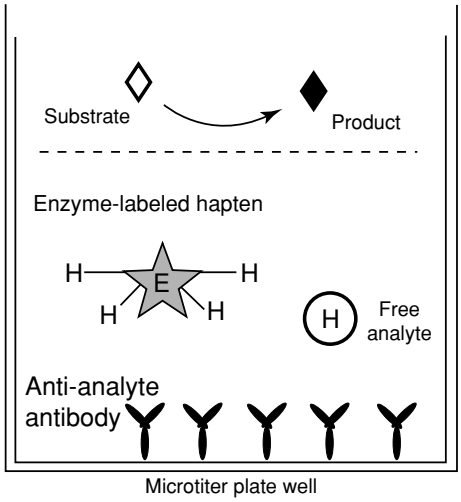


Figure 3 Immobilized antibody ELISA. Primary antibody (**Y**) is passively adsorbed to the surface of a polystyrene microtiter plate. Analyte (free **H**) and an enzyme-labeled hapten (**H**–**E**) compete for the fixed number of primary antibody binding sites. Following a wash step (dotted line), the substrate for the enzyme is added (◇) and a colored product formed (◆). The amount of product is inversely proportional to the amount of analyte present

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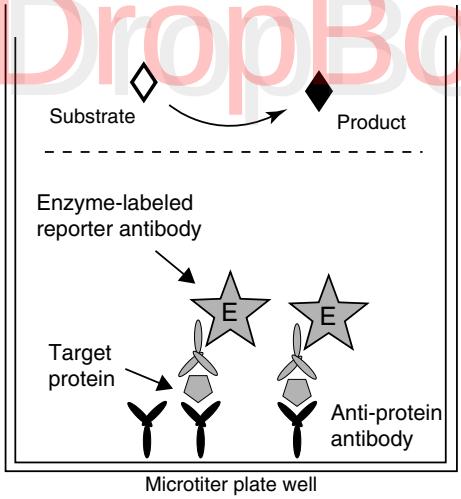


Figure 4 Sandwich immunoassay. A capture antibody (**Y**) is passively adsorbed on a solid phase. The target protein contained in the sample and the enzyme-labeled reporter antibody (**Y**–**E**) are added. Both the capture antibody and enzyme-labeled reporter antibody bind to the target protein at different sites, 'sandwiching' it between the antibodies. Following a wash step, the substrate (◇) is added and colored product (◆) formed. The amount of colored product is directly proportional to the amount of target protein captured

binding occurs. A second, analyte-specific, enzyme-labeled antibody is added and it also binds to the analyte, forming a sandwich. A substrate is added, producing a colored product. Unlike the competitive immunoassays described in Figures 2 and 3, the absorbance in the sandwich immunoassay is directly proportional to the concentration of the analyte in the sample solution.

A commonly used field-portable immunoassay format is the lateral flow device. Lateral flow devices are designed for threshold or qualitative testing. Advantages of this format are that the cost per test is low, it is field portable, it can be done at ambient temperature, it requires no specialized equipment and only minimal user training is required. Each immunoassay strip test (lateral flow device) is a single unit allowing for manual testing of an individual sample. The device contains a reporter antibody labeled with a colored particle such as colloidal gold or latex, which is deposited in a reservoir pad. An analyte-specific capture antibody is immobilized on the membrane. When the strip is placed into the test solution, the solution enters the reservoir pad and solubilizes the labeled reporter antibody, which binds to the target analyte. This analyte–antibody complex flows with the liquid sample laterally along the surface of the strip. When the complex passes over the zone where the capture antibody has been immobilized, the complex binds to the capture antibody and is trapped, accumulating and producing the appearance of a colored band at the capture zone on the strip. If the result is negative and no analyte is present in the test solution, only the control band appears in the result window. This band indicates that the liquid flowed properly up the strip. If the result is positive, two bands appear in the result window. A lateral flow strip test can provide a yes/no determination of the presence of the target analyte or a threshold (semi-quantitative) result, typically in 5–10 min.

Commercial test kits that use 96-well microtiter plates or test tubes have been available for some pesticides since the 1980s.[16] Several vendors have assays for analytes such as herbicides that appear in groundwater or runoff water, e.g., triazines, alachlor, diazinon and chlorpyrifos. More recent emphasis has been the production of kits for compounds of concern in developing countries (such as DDT) and for GMOs. When selecting a test kit, the user should determine the intended use, (i.e., as a screening method or a quantitative method) and whether the method will be used in the laboratory or the field. The cost per assay, assay sensitivity, cross-reactivity, availability of published validation by independent groups and the availability of technical support are important considerations in selecting a test kit. It is critical that the assay has been validated in the matrix of interest. If a kit or method intended for water is used for another aqueous media such as urine, inaccurate results may be obtained. Because the test kit must be validated in the matrix of concern, the sponsoring company will usually actively collaborate or assist with the validation. Several papers on test kit validations or comparisons of test kits from different manufacturers have been published.[16–19]

2.3 Data reduction

The absorbance values obtained are plotted on the ordinate (linear scale) against the concentration of the standards on the abscissa (logarithmic scale), which produces a sigmoidal dose–response curve (Figure 5). The sigmoidal curve is constructed by

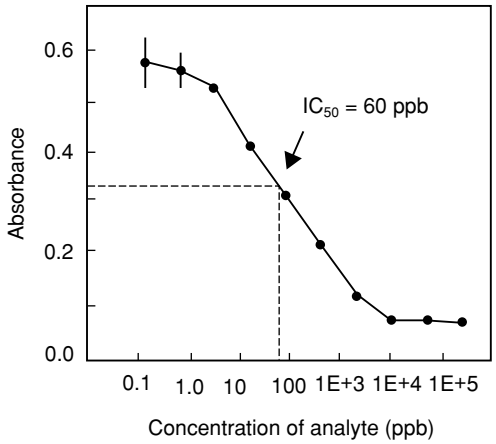


Figure 5 An example calibration curve. Absorbance is plotted against log (concentration of analyte). The competitive equilibrium binding process results in a sigmoidal curve that is fitted using a four-parameter fit. [20] The IC_{50} is defined as the concentration of analyte that results in a 50% inhibition of the absorbance

using the four-parameter logistic curve regression of the known concentration of the standard calibration solutions and their subsequent absorbance.[20]

Assay sensitivity is defined here as the concentration of analyte that inhibits the observed absorbance by 50% or the IC_{50}. The lower limit of detection (LLD) is the lowest analyte concentration that elicits a detector response significantly different from the detector response in the absence of analyte. In some cases, the LLD is defined as three standard deviations from the mean of the zero analyte control. In other cases, the LLD is defined empirically by determining the lowest concentration of analyte that can be measured with a given degree of accuracy. Readers are referred to Grotjan and Keel[21] for a simplified explanation and to Rodbard[22] for the complete mathematics on the determination of LLD.

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The concentration of analyte in the unknown sample is extrapolated from the calibration curve. To obtain an accurate and precise quantitative value, the optical density (OD) for the sample solutions must fall on the linear portion of the calibration curve. If the sample OD is too high, the sample solution must be diluted until the OD falls within the quantitative range of the assay. The concentration of the analyte in the original sample is calculated by correcting for any dilution factor that was introduced in preparing the sample for application to the microplate.

2.4 Sample collection and preparation

Once the immunoassay that meets the study objectives has been identified, sample collection begins. Proper sampling is critical in order to obtain meaningful results from any type of analytical assay. An appropriate sampling scheme will support the objective of the test. For example, a plant breeder may take a single leaf punch to determine quickly whether a specific protein has been expressed in an experimental plant. A more complex sampling regime would be used to determine the expression

profile of a specific protein in corn grain, leaves and stalks for a regulatory study. These regulatory field studies are often modeled after crop residue studies for chemical pesticides. The protocol typically describes sampling from representative plants, tissues, growth stages and geographical sites.

Sampling has the potential to introduce significant uncertainty and error into a measurement; therefore, a proper plan should be devised with the assistance of a qualified statistician. Grain sampling is a routine practice and standard methods for taking samples from static lots – such as trucks, barges and railcars – and for taking samples from grain streams can be found in the United States Department of Agriculture Grain Inspection Protection Service (USDA GIPSA) 'Grain Inspection Handbook, Book 1, Grain Sampling'.[23] Ultimately, the optimum sampling strategy is a balance between sensitivity, cost and confidence.

Sample preparation techniques vary depending on the analyte and the matrix. An advantage of immunoassays is that less sample preparation is often needed prior to analysis. Because the ELISA is conducted in an aqueous system, aqueous samples such as groundwater may be analyzed directly in the immunoassay or following dilution in a buffer solution. For soil, plant material or complex water samples (e.g., sewage effluent), the analyte must be extracted from the matrix. The extraction method must meet performance criteria such as recovery, reproducibility and ruggedness, and ultimately the analyte must be in a solution that is aqueous or in a water-miscible solvent. For chemical analytes such as pesticides, a simple extraction with methanol may be suitable. At the other extreme, multiple extractions, column cleanup and finally solvent exchange may be necessary to extract the analyte into a solution that is free of matrix interference.

The protein analyte is extracted from the plant material by adding a solvent and blending, agitating or applying shearing or sonic forces. Typical solvents used are water or buffered salt solutions. Sometimes detergents or surfactants are added. As with chemical pesticide extraction methods, the protein extraction procedure must be optimized for the specific sample matrix. Processed samples may have been subjected to processes resulting in protein precipitation and/or denaturation. These factors can influence protein extraction efficiency. The problem can often be overcome by changing the buffer composition and the extraction procedure.

Because the protein analyte is endogenous to the plant, it can be difficult to demonstrate the efficiency of the extraction procedure. Ideally, an alternative detection method (e.g., Western blotting) is used for comparison with the immunoassay results. Another approach to addressing extraction efficiency is to demonstrate the recovery of each type of protein analyte from each type of food fraction by exhaustive extraction, i.e., repeatedly extracting the sample until no more of the protein is detected.[24]

Some examples are given below to illustrate extraction procedures for proteins that have been optimized for different matrices and testing strategies.

Neomycin phosphotransferase II (NPTII) extraction from cotton leaves and cottonseed. The extraction buffer consists of 100 mM Tris, 10 mM sodium borate, 5 mM magnesium chloride, 0.2% ascorbate and 0.05% Tween 20 at pH 7.8. The frozen leaf sample is homogenized in cold (4 °C) buffer. An aliquot of the homogenate is transferred to a microfuge tube and centrifuged at 12 000 *g* for 15 min. The supernatant is diluted and assayed directly by ELISA.

The extraction procedure for cottonseed samples is the same, except that the cottonseed samples are crushed before the buffer is added for homogenization.[25]

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) extraction from processed soybean fractions. The extraction buffer consists of 0.138 M NaCl, 0.081 M Na_2HPO_4, 0.015 M KH_2PO_4, 0.027 M KCl and 2% sodium dodecyl sulfate (SDS) at pH 7.4. Aqueous buffers are inadequate to extract EPSPS efficiently from processed soybean fractions owing to protein precipitation and the denaturation that occurs throughout soybean processing. Efficient extraction is achieved through the use of detergent in an aqueous buffer, mechanical tissue disruption and heating.[25]

Bt11 endotoxin extraction from corn grain. The following example is a description of a commercial kit procedure for extraction of the Cry1A (b) and Cry1A (c) from corn grain for analysis with an immunoassay strip test (lateral flow device). It is important to note that for the Bt11 event the endotoxin is expressed in seed (grain) and plant tissue. However, corn plants from the Bt176 event do not express detectable quantities of the *Bacillus thuringiensis* (Bt) endotoxin in grain, and therefore a negative result in a corn grain sample does not necessarily mean the sample does not contain genetically modified material.

Reagents A and B are supplied with the kit, but the composition of these solutions is not described. A sample (25 g) of corn grain is weighed into a 4-oz glass Mason jar. Using a Waring blender, the sample is ground for 10 s on the low-speed setting. Buffered water (40 mL), consisting of 200 mL of Reagent A in 1 gal of distilled water, is added to the ground corn. The jar is capped and shaken vigorously for at least 30 s. The solids are allowed to settle and the supernatant is withdrawn with a transfer pipet. Six drops of the supernatant are dispensed into the reaction tube and three drops of Reagent B are added. The reaction tube is capped and mixed by inverting it three times. The sample is analyzed with the lateral flow device.[26]

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2.5 *Development of pesticide immunoassays*

The development of sensitive and inexpensive immunoassays for low molecular weight pesticides has been an important trend in environmental and analytical sciences during the past two decades.[8,10,27–29] To design an immunoassay for a pesticide, one can rely on the immunoassay literature for agrochemicals,[30–32] but many of the innovations in clinical immunoanalysis are also directly applicable to environmental analysis.[11,33,34] Conversely, the exquisite sensitivity required and difficult matrices present for many environmental immunoassay applications have forced the development of technologies that are also useful in clinical immunoassay applications. In the following discussion we will describe widely accepted procedures for the development of pesticide immunoassays.

The major steps in the development of an immunoassay are as follows:

- design and synthesis of haptens
- conjugation of haptens to antigenic macromolecular carriers
- immunization of host animals and subsequent generation of antibodies

- characterization of antibodies
- assay optimization
- assay validation.

2.5.1 Basic analysis of the target analyte structure

In general, immunoassays are more readily developed when the target analyte is large, hydrophilic, chemically stable and foreign to the host animal.[8] In theory, the sensitivity and selectivity of an immunoassay are determined by the affinity of the antibody to the analyte, and hence immunogen design and antibody production are of fundamental importance to assay development. For a molecule to be immunogenic it must have a molecular mass of at least 2000 Da and possess a complex and stable tertiary structure. Low molecular weight antigens (less than 2000 Da), a size that includes most pesticides, are not directly immunogenic. Such nonimmunogenic molecules are termed 'haptens'. Haptens possess no, or very few, epitopes that are recognizable by immune systems of host animals. As a consequence, they must be linked to larger molecules in order to become immunogenic to host animals.

Factors an analyst should consider when designing a hapten–immunogen system are outlined in Table 1. The immunizing hapten should be designed to mimic closely the target analyte. Ideal haptens have close chemical similarity to the target analyte and possess a functional group to allow coupling to carrier molecules; coupling to carrier antigens usually occurs through a 'linker,' 'spacer' or 'handle' molecule (discussed below). Retention of the unique functional groups of the analyte, especially ionizable groups or groups that form hydrogen bonds, are critical for the production of high-affinity antibodies. Also important are the ease of hapten synthesis, hapten solubility, and the nature of the method to be used for conjugation to proteins.

2.5.2 Design of the immunizing hapten

(1) Position of spacer arm. The position of the linker group on the target analyte that connects it to the immunogen has a profound influence on the selectivity and sensitivity of the subsequent assays. The handle should be attached as far as possible from the unique determinant groups, allowing maximum exposure of the important

Table 1 Guidelines for the design and synthesis of an immunogen hapten

1. Position of handle on target molecule
Distal to hapten determinant groups
Avoid attachment to functional groups
2. Handle selection
Length of handle
Avoid functional groups in handle (unless used to increase exposure or improve solubility)
3. Coupling of haptens
Type of coupling reaction
Compatibility of reaction with target molecule functional groups
4. Stability of hapten under coupling conditions and subsequent use
5. Ease of synthesis
6. Characterization of conjugates and determination of hapten/protein ratio

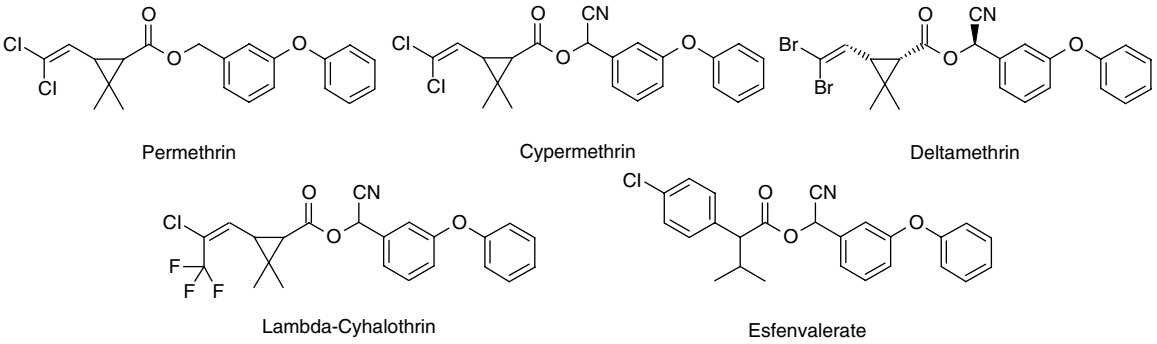


Figure 6 Structures of some major use pyrethroids

structural features of the analyte to the immune system. Presentation of unique features of the target analyte is particularly important for ensuring selectivity to a single chemical structure within a chemical class. For example, we attempted to develop compound-specific immunoassays for the major pyrethroids esfenvalerate, permethrin and cypermethrin. As shown in Figure 6, these pyrethroids have similar or identical alcohol moieties, while containing relatively unique acyl substituents. If a carrier protein was linked through the acid portion, leaving the common phenoxybenzyl group unchanged, the resulting antibodies generated from such an immunogen would be expected to recognize many pyrethroids. In order to develop a compound-specific assay, we retained the relatively unique acid substituents, and attached the linkers to the aromatic phenoxy benzyl groups (Figure 7). Using this strategy, sensitive and selective assays for permethrin and esfenvalerate were developed.[35,36] Another design option was to modify the α-cyano group to support a linker for protein conjugation (Figure 8). In this case, nearly the whole pyrethroid is unchanged; antibodies developed based on this strategy were specific for the target compounds.[37,38]

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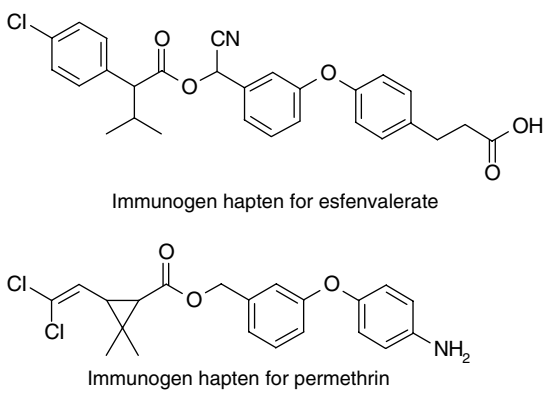


Figure 7 Structure of the haptens used in the immunogen for the development of antibodies that recognize pyrethroid insecticides, esfenvalerate and permethrin. The esfenvalerate hapten was coupled to proteins through the carboxylic acid group and the permethrin hapten was coupled to proteins through the amine group. Because antibody recognition of the structure is greatest most distal to the point of attachment to the protein, the antibodies were selective for the acid portions of the pyrethroid molecules resulting in highly selective assays for esfenvalerate and permethrin, respectively

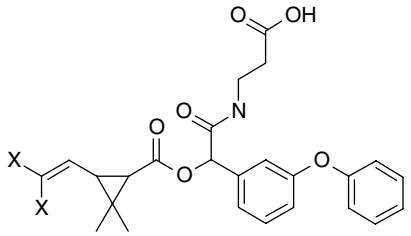


Figure 8 Structure of immunogen haptens for pyrethroids with spacer arm attachment at the α-position of the alcohol moiety. Since the whole pyrethroid molecule is available for recognition by the antibody, assays resulting from these immunogens were selective for the parent pyrethroids

However, if a class-selective assay is desirable (for multi-analyte assays), the handle should be located at or near a position that differentiates members of the class and exposes features common to the class. Using the pyrethroid example, an ideal immunogen should retain the phenoxybenzyl moiety and link the protein from the distal acid end (Figure 9). Using such an immunogen hapten, a class-specific immunoassay was developed that was highly cross-reactive with the type I pyrethroids permethrin, phenothrin, resmethrin and bioresmethrin.[39]

For small molecules, the retention of each determinant group identity is very important. Attaching the handle to a determinant group should be avoided because this alters the target molecule's structure, geometry and electronic properties relative to the parent compound. Some target analytes may contain acid, amino, phenol or alcohol groups that can be directly conjugated. Because hydrogen bonding is often the major force for interaction between an antigen and an antibody, such groups are very important determinants for antibody affinity and specificity. A good example of functional group importance is the immunoassay for phenoxybenzoic acid (PBA), a major metabolite of some pyrethroids. To develop an antibody against PBA, two options were used to design and conjugate haptens to the carrier protein. Phenoxybenzoic acid was directly conjugated with the antigenic protein using its −COOH group (Figure 10, site 2). This reaction could be accomplished using relatively simple chemistry for conjugation, but would likely result in poor antibody specificity because the phenoxybenzyl moiety is present in many parent pyrethroids. In addition,

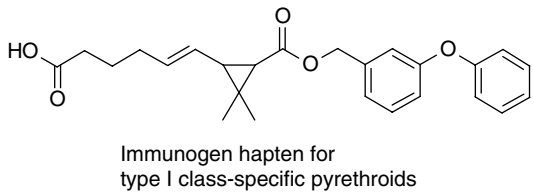
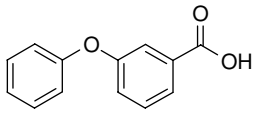


Figure 9 Structure of the immunogen hapten used to generate antibodies for a type I pyrethroid class-selective assay. Pyrethroids lacking an α-cyano group are generally termed type I. This hapten exposed the features most common to type I pyrethroids, the phenoxybenzyl group, the cyclopropyl group and the lack of a cyano group, resulting in antibodies that recognized permethrin, phenothrin, resmethrin and bioresmethrin, but not cypermethrin



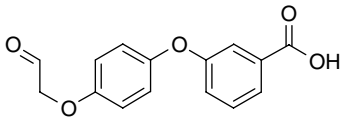
Phenoxybenzoic acid (PBA)

Figure 10 Structure of the target analyte phenoxybenzoic acid (PBA). The arrows point to the ideal sites for conjugation of the molecule to proteins for optimum recognition. Use of site 2 for conjugation to protein resulted in antibodies that recognized free PBA poorly

the lack of hydrogen bonding elements and reduced solubility of conjugates would likely significantly influence subsequent antibody affinity and specificity. Alternatively, we designed a hapten that left the −COOH group unchanged by attaching to the distal aromatic benzene, site 1, a linker containing a terminal aldehyde group that was used to conjugate to protein (Figure 11). The resulting antibodies had a high binding affinity and resulted in the development of a highly sensitive and selective assay [($IC_{50} = 1\ \mu g\ L^{-1}$ (ppb)] that was about 1000 times more sensitive than the assay developed from an antibody raised against an immunogen conjugated at site 2. No cross-reactivity to any other parent pyrethroid or their metabolites was measured for the antibody resulting from site 1 conjugation. Although some structural change in the target molecule is usually unavoidable, when selecting a handle for the immunogen hapten the original steric and electronic characteristics of the target molecule should be preserved as much as practical. Especially electronic features including electron density around important atoms, net charge at important atoms and hybridization of electronic orbitals of characteristic groups should be preserved.

(2) Handle selection. For small molecules (including most pesticides), the selection of a spacer or linker arm is important. Omitting the spacer arm from the structure of immunogen may result in assays with poor sensitivity and/or weak recognition of the portion of the target molecule near the attachment to the carrier protein. Generally, the optimal linking group has a chain length of about four to six atoms.[40–42] For hydrophobic haptens such as pyrethroids and dioxins, the role of the spacer may be of critical importance because the hapten may fold back on the protein surface or within the protein core after conjugation. The antibody resulting from such an immunogen will have low affinity and poor selectivity. A hapten with a rigid spacer can overcome such hydrophobic interactions. A double bond-containing spacer for the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) immunogen hapten (Figure 12) resulted in a highly sensitive immunoassay with an IC_{50} of 240 ng L^{-1}.[43,44] In contrast, when a flexible hexanoic acid spacer was used for development of an ELISA

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Immunogen hapten for PBA

Figure 11 Structure of the phenoxybenzoic acid (PBA) immunogen hapten. Conjugation to the protein through the aldehyde resulted in an immunogen that generated antibodies selective and sensitive for PBA

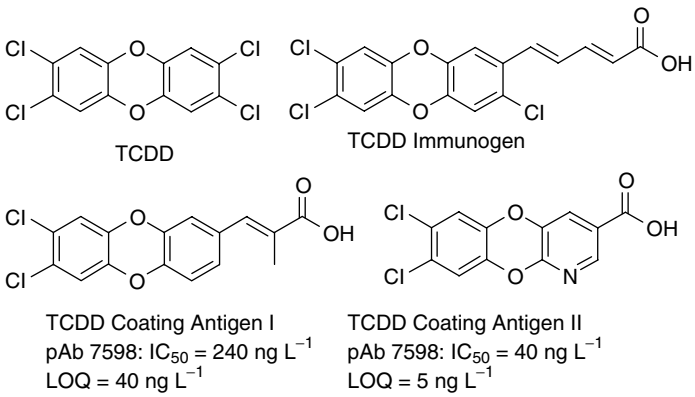

TCDD
TCDD Immunogen
TCDD Coating Antigen I
pAb 7598: IC50 = 240 ng L−1
LOQ = 40 ng L−1
TCDD Coating Antigen II
pAb 7598: IC50 = 40 ng L−1
LOQ = 5 ng L−1

Figure 12 Structures of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), immunogen and coating haptens. The immunogen was synthesized with a rigid spacer so the lipophilic hapten would not fold back into the hydrophobic core of the protein preventing recognition by the immune system. The affinity of the antibody for coating antigen II is less than for coating antigen I owing to structural changes, hence the assay using coating antigen II is more sensitive for TCDD

for polychlorinated biphenyls (PCBs), a modestly successful assay with an IC_{50} of 100 μg L^{-1} resulted.[45]

In concept, a lipophilic hapten can be attached to glycoprotein linkers to prevent the hapten from folding into the protein. However, the use of glycoprotein linkers may lead to the recognition of the handle. In general, the spacer arm should not include polar, aromatic or bulky groups; at a minimum, these moieties should not be linked directly to the target structure. An aliphatic straight-chain linker is preferred.[46]

2.5.3 Haptens for coating antigens and tracers

Careful design of coating haptens should take into consideration the reversible antibody/analyte equilibrium competition with an antibody/hapten–protein conjugate that is illustrated in Figure 1. Assuming that no analyte (**A**) is present, only the K_H, which is variable by changing hapten structure, for coating hapten–protein (**H**) is in operation between antibody (**Y**) and coating antigen (**H**), and a maximum signal from the **Y–H** is observed. On the addition of analyte (**A**), this equilibrium is shifted towards the formation of antibody–analyte (**Y–A**), described by K_A. Formation of **Y–A** dramatically reduces the amount of **Y–H** and hence the tracer signal decreases. Thus, for a fixed quantity of antibody; the lowest IC_{50} (or sensitivity) is observed when the affinity of the antibody for the analyte is greater than the affinity of the antibody for the coating-hapten ($K_A \gg K_H$). Therefore, with a fixed K_A for **Y–A**, one can shift the equilibrium by selecting a coating hapten with decreased relative affinity for the antibody; lower analyte concentrations may compete with these reagents under equilibrium conditions, resulting in assays with greater sensitivities. This competition is the rationale for improving assay sensitivity through use of heterologous haptens[47] and is employed extensively in our laboratory for triazine herbicides,[41,48] arylurea herbicides,[46,49] pyrethroid insecticides[35,36,39] and dioxins.[44,50] Guidelines for obtaining this heterology are outlined in Table 2.

Table 2 Guidelines for design of coating/tracer haptens

1. Heterology of hapten structure
Position of handle
Composition of handle
Conjugation chemistry
2. Alterations in target molecule structure
Use of partial structure
Change of key determinants
3. Cross-reactivity data of hapten structures (or derivatives)
4. Determination of hapten/protein ratio

Hapten heterology, site heterology, linker heterology, geometric heterology and the use of different conjugation techniques (discussed later) are useful tools to improve assay performance for both coating-antigen and enzyme tracer formats. In the development of TCDD immunoassays, our first assay employed a heterologous hapten **I** containing a short linker that lacked chlorine at position 2; a sensitive immunoassay resulted.[44] To improve the sensitivity, a new coating antigen (hapten **II**) was designed by replacing the benzene ring proximal to the linker with a pyridine ring (Figure 12). The resulting assay was five times more sensitive than the original assay having an IC_{50} of 40 ng L^{-1} and a limit of quantitation (LOQ) of 5 ng L^{-1}.[50]

Immunoassays for diuron (Figure 13) are another example of improved assay performance using heterologous assay conditions. One antibody was derived from a hapten that extended the dimethylamine side chain of diuron with methylene groups.

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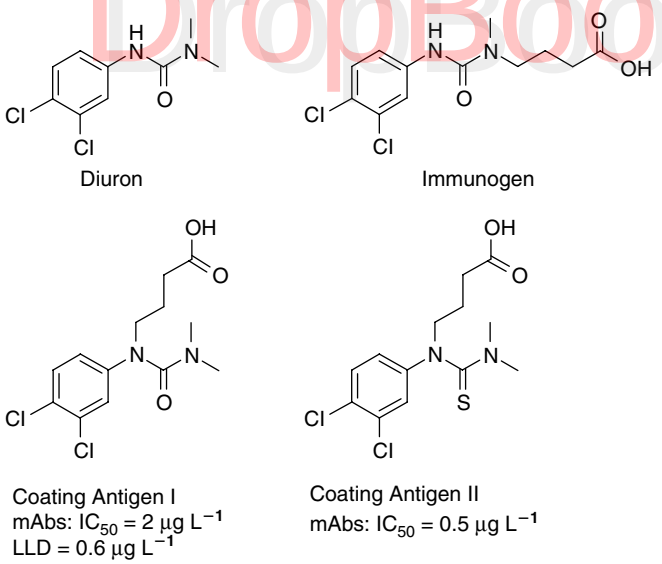
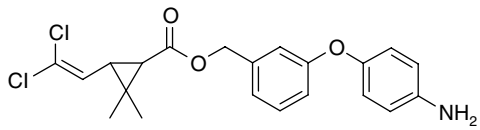


Figure 13 Structures of haptens used for immunizing and coating antigens in a monoclonal antibody-based immunoassay for diuron. A sensitive assay was developed using coating hapten I that had the handle in a position different from the immunogen hapten. When the oxygen in the urea moiety of hapten I was replaced with a sulfur (hapten II), increasing the heterology, even greater sensitivity was achieved

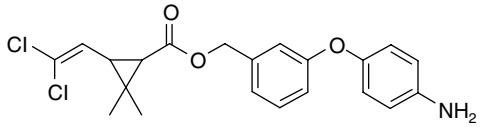
The best coating antigen of three evaluated consisted of an isomer in which the butyric acid handle was attached to the dichloroaniline nitrogen. The IC_{50} was 2 μg L^{-1} with an LOQ of 0.6 μg L^{-1}.[49] Using the rationale that a coating hapten with a lower affinity for the antibody was desirable, we replaced the oxygen of the diuron immunogen hapten with a sulfur to make a thiourea coating antigen. The resulting assay had an IC_{50} of 0.5 μg L^{-1} for diuron.[46] Sulfur, being larger than oxygen, probably did not fit well in the anti-diuron antibody pocket and there would be a substantially lower affinity owing to the loss of hydrogen bonding between the thiocarbonyl and antibody.

For chiral haptens, the use of enantiomers or diastereoisomers as the coating hapten may significantly improve the assay sensitivity. This was the case in the development of the permethrin immunoassay. The antibody was raised against a *trans*-permethrin hapten (Figure 14). Use of the corresponding *cis*-permethrin hapten as a coating antigen resulted in a sensitive and selective assay with an IC_{50} of 2.5 μg L^{-1} and an LOQ of 0.4 μg L^{-1}, which is about 200 times more sensitive than the homologous system in which the *trans*-permethrin hapten was the coating antigen.[35]

There are tradeoffs with developing assays based on assay heterology. For example, the highest titer of antibody is normally identified with a coating hapten that is very similar to the immunizing hapten. Rabbit antisera raised against acylurea insecticide haptens had high titers for the acylurea haptens that were similar to the immunizing structure. However, the target acylurea insecticide could not inhibit these assays because the antibodies bound to the coating hapten with greater affinity than to the acylurea insecticide. Changing the coating hapten to one containing a different handle than used for the immunizing hapten resulted in a decrease in antibody titer, demonstrating that the antibody bound with less affinity to the new coating antigen. However, the affinity for the target analyte was improved and a very sensitive assay for the acylurea insecticides resulted.[47] The benefit of careful design of a heterologous assay normally is greater with small haptens and spacers (primary or secondary amines compared with tertiary amines and amides) that are readily distinguished by the immune system than it is with large haptens.



trans-Permethrin hapten



cis-Permethrin hapten

Figure 14 Permethrin immunogen and coating antigen haptens. Using enantiomers or diastereoisomers is a strategy to provide hapten heterology. Assays using antibodies raised to the *trans*-permethrin hapten were more sensitive when the *cis*-permethrin hapten was used instead of the *trans*-permethrin hapten for the coating antigen

2.5.4 Hapten conjugation

In order to elicit a satisfactory immune response, haptens must first be covalently attached to a carrier protein, which is usually foreign to the animal being immunized. In addition, the hapten used for immunization and other similar haptens are conjugated to enzymes and (or) other proteins for use in the assay. For hapten–protein conjugates, protein solubility, the presence of functional groups and stability under reaction conditions are important variables to consider during immunoassay development. Many conjugation methods are available[14,51–53] and the selection of an appropriate method is ultimately dependent on the functional group available in the hapten.

(1) Carrier protein. A wide variety of proteins are available for the synthesis of immunogens or antigens including bovine serum albumin (BSA) and human serum albumin (HSA), ovalbumin, thyroglobulin, keyhole limpet hemocyanin (KLH) or horseshoe crab hemocyanin (LPH), and the synthetic polypeptides poly-L-lysine and polyglutamic acid. Among these, KLH is often the first choice as an immunogen carrier protein because it is large (approximately 10^6 Da) and is highly immunogenic. In addition, KLH contains an abundance of functional groups available for conjugation, including over 2000 lysine amines, over 700 cysteine sulfhydryls and over 1900 tyrosine residues. It should be noted that KLH requires a high-salt buffer (at least 0.9 M NaCl) to maintain its stability and solubility. In solutions with NaCl, concentrations lower than 0.6 M KLH will precipitate and denature, and maintaining solubility after hapten conjugation can be difficult. Hence conjugation reactions using KLH should be carried out under high-salt conditions to preserve the solubility of the hapten–carrier complex.

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Thyroglobulin has been increasingly used as an immunogenic carrier protein owing to its excellent water solubility. Another frequently used protein in immunoassay is BSA. Although BSA is immunogenic, it is mostly used as a coating antigen carrier. Advantages of BSA include its wide availability in relatively pure form, its low cost and the fact that it is well characterized. BSA has a molecular weight of 64 000 and it contains 59 primary amino groups, one free cysteine sulfhydryl, 19 tyrosine phenolate residues and 17 histidine imidazolides. It is also relatively resistant to denaturation and is suitable for some conjugation procedures that involve organic solvents. Moreover, BSA conjugates are usually readily soluble, which makes their isolation and characterization easier. Although a general rule states that large and phylogenetically foreign proteins make the best antigenic proteins, we have obtained antibodies when smaller proteins such as fetuin were used as carriers.[54]

(2) Conjugation methods. The selection of conjugation method is dependent on the functional group on the hapten (e.g., carboxylic acid, amine, aldehyde). A hapten with a carboxylic acid group can conjugate with a primary amino group of a protein using the carbodiimide, activated *N*-hydroxysuccinimide (NHS) ester or mixed anhydride methods. Haptens with free amines can be coupled to proteins using glutaraldehyde condensation or diazotization. Haptens that have been designed to contain spacers may be linked directly to the protein with methods such as the mixed anhydride, whereas haptens lacking a spacer should be coupled using methods that insert a linker between the hapten and the protein such as with glutaraldehyde. Typical procedures

Table 3 Conjugation of a carboxyl-containing hapten to a protein using a carbodiimide method

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- EDC[a]
- Phosphate buffer (0.1 M, pH 6): prepared from KH_2PO_4 (3.025 g), Na_2HPO_4 (0.39 g) and water (250 mL)

Method
1. Dissolve the hapten (0.04 mmol) in phosphate buffer containing 50 mg of BSA
2. Add 150 mg (0.78 mmol) of EDC to the buffer solution. Stir the mixture at room temperature to allow all the reagents to dissolve
3. React at room temperature for 24 h
4. Purify conjugate by gel filtration, dialysis or ethanol precipitation

[a] EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl.

are provided below for methods that have been successfully used in this laboratory or for which extensive literature is available.

(3) Haptens with free carboxylic acids. Methods for linking hapten carboxyl groups to amine groups of antigenic proteins include activation by carbodiimides, isobutyl chloroformate or carbonyldiimidazole. In the widely used carbodiimide method, the carbodiimide activates the carboxylic acid to speed up its reaction with the amine. Acidic conditions catalyze the formation of the active *O*-acylurea intermediate while the protein is more reactive at higher pH, when the lysine amino groups are unprotonated. Therefore, as a compromise, a pH near 6 is used. The choice of carbodiimide is dependent on the reaction conditions. For example, dicyclohexylcarbodiimide (DCC) is used in nonaqueous media with nonpolar, water-insoluble haptens where the carrier protein, in aqueous solution, is added to the activated hapten in a two-step reaction. For more water-soluble haptens, water-soluble derivatives of DCC such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC or Morpho CDI) are used in one-step reactions (Table 3, Figure 15). However, EDC will react directly with protein, and some antibodies are certain to be

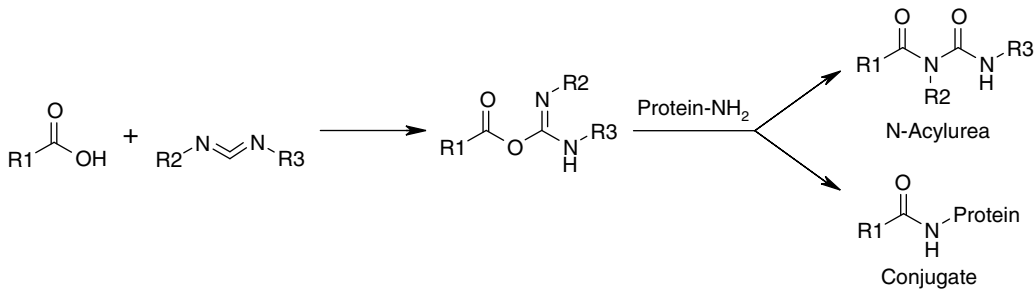


Figure 15 Conjugation of a carboxylic acid and an amine using the carbodiimide method. The carbodiimide activates the carboxylic acid to speed up the reaction to the amine. Carbodiimides can be used with nonpolar or polar solvents, including water. Undesirable urea complexes may form as by-products. Details of the reaction are given in Table 3

Table 4 Conjugation of a carboxyl-containing hapten to a protein using *N*-hydroxysuccinimide

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- DCC
- NHS
- DMF[a]
- Phosphate buffer (0.1 M, pH 7.4): prepared from KH_2PO_4 (0.67 g), Na_2HPO_4 (0.285 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.04 mmol) in DMF (0.5 mL)
2. Add DCC (15 mg, 0.15 mmol) followed by NHS (20 mg, 0.17 mmol)
3. React at room temperature for 3.5 h
4. Remove the precipitate, dicyclohexylurea, by centrifugation
5. Add the supernatant to phosphate buffer (~5 mL) containing 50 mg of BSA
6. React at room temperature for 2 h
7. Purify conjugate by gel filtration, dialysis or ethanol precipitation

[a] DMF = dimethylformamide (>99%, from Aldrich).

generated to the resulting highly immunogenic protein–urea complex. Formation of these antibodies is not a drawback as long as a different coupling chemistry is used to prepare coating antigens.

Activated NHS esters of carboxylic acids are prepared by reacting the acid with NHS in the presence of DCC (Table 4, Figure 16). *N*-Hydroxysuccinimide esters are stable when kept under anhydrous and slightly acidic conditions, and they react rapidly with amino groups to form an amide in high yield.

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Like the carbodiimide method, the mixed anhydride method[55,56] results in an amide complex (Table 5, Figure 17). The acid-containing hapten is dissolved in a dry, inert, dipolar, aprotic solvent such as *p*-dioxane, and isobutyl chloroformate is added with an amine catalyst. The activated mixed anhydride is chemically stable and can be isolated and characterized. The aqueous protein solution is added to the activated acid and the pH is maintained at around 8.5. A low temperature (around 10 °C) is necessary during the reaction to minimize side reactions.

(4) Haptens with an amino group. Amine groups in haptens, carrier proteins or both can be modified for conjugation through homo- or heterobifunctional cross-linkers such as acid anhydrides (e.g., succinic anhydride), diacid chlorides (e.g.,

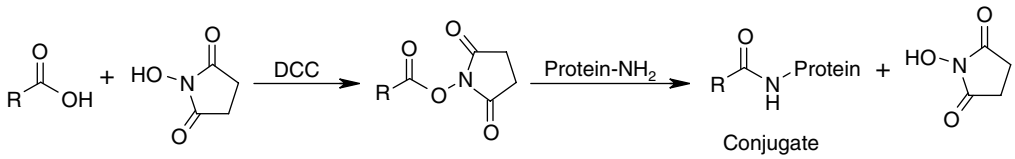


Figure 16 Conjugation of an amine and a carboxylic acid via the *N*-hydroxysuccinimide (NHS)-activated ester method. NHS esters may be isolated and characterized and are stable to long term storage as the powder. Alternatively, the NHS esters may be used immediately upon formation without isolation. Details of the reaction are given in Table 4

Table 5 Conjugation of a carboxyl-containing hapten to a protein using the mixed anhydride procedure

Materials

BSA (Sigma, Fraction V or similar)
Hapten
Isobutyl chloroformate
1,4-Dioxane (>99%, from Aldrich)
Tributylamine

Method

1. Dissolve the hapten (0.04 mmol) in dioxane (5 mL) in a small tube and cool to 10 °C
2. Add tributylamine (11 μL, 0.044 mmol) to the solution followed by isobutyl chloroformate (6 μL, 0.044 mmol)
3. React at 10 °C for 60 min to activate the carboxylic acid
4. Add BSA solution (50 mg of BSA dissolved in 5 mL of distilled water and adjusted to pH 9 with NaOH) and stir for 4 h
5. Monitor the solution pH over the period and maintain it at 8.5 by the addition of dilute NaOH
6. Purify conjugate by gel filtration, dialysis or ethanol precipitation

succinyl chloride) or dialdehydes (e.g., glutaraldehyde). Glutaraldehyde condensation (Table 6) has been used widely to produce protein–protein and hapten–protein conjugates. The glutaraldehyde reagent should not have undergone polymerization. To check for polymerization, add a few drops of water to an aliquot of stock glutaraldehyde solution; a white precipitate is indicative of polymerization whereas unpolymerized reagent will not precipitate.

A disadvantage of the glutaraldehyde condensation method is that dimers of the hapten and polymers of carrier protein may also form. To overcome this problem, the reaction time is limited to 2–3 h, or an excess of an amine-containing compound, e.g., lysine or cysteamine hydrochloride, is added. A two-step approach also minimizes dimerization.[57]

Aromatic amine-containing haptens are converted to diazonium salts with ice-cold nitrous acid. Diazonium salts can then react with a protein at alkaline pH (around 9) through electrophilic attack of the diazonium salt at histidine, tyrosine and(or) tryptophan residues of the carrier protein (Table 7).

(5) Other reactions. Other reactions can also be used to couple haptens to proteins. The periodate oxidation is suitable for compounds possessing vicinal hydroxyl groups such as some sugars. Schiff's base method has been used for conjugating aldehyde-containing haptens to primary amino groups of carrier proteins. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) is a heterobifunctional reagent that will cross-link a free amine at one end and a free thiol at the other. Heterobifunctional

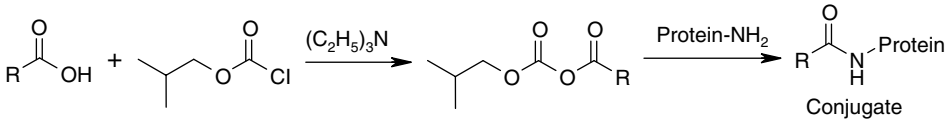


Figure 17 Conjugation of an amine and a carboxylic acid via the mixed anhydride method. Although the activated mixed anhydride is stable, it is usually used without purification. Use of low-temperature reactions will limit undesirable side products. Details of the reaction are given in Table 5

Table 6 Conjugation of an amino-containing hapten to a protein using the glutaraldehyde method

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- Glutaraldehyde solution (0.2%, 0.02 M) in buffer
- Lysine monohydrochloride (1 M) in water
- Phosphate buffer (0.1 M, pH 7): prepared from KH_2PO_4 (1.40 g), Na_2HPO_4 (2.04 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.03 mmol) and BSA (40 mg) in phosphate buffer
2. Add the glutaraldehyde solution (2 mL) dropwise over a period of 30 min
3. React at room temperature for 90 min. During this period the reaction mixture should turn yellow
4. Add the lysine solution to quench the reaction and stir for 60 min
5. Purify conjugate by gel filtration, dialysis or ethanol precipitation

reagents are commercially available but their use for immunizing antigens may lead to extensive handle recognition. A more complete discussion of other cross-linking and conjugation reagents can be found in Hermanson.[51]

2.5.5 Characterization of conjugates

Hapten density is important for both immunization and assay performance, and hence the extent of conjugation or hapten density should be confirmed by established methods. A characteristic ultraviolet (UV) or visible absorbance spectrum that distinguishes the hapten from the carrier protein or use of a radiolabeled hapten can be used to determine the degree of conjugation. If the hapten has a similar λ_{max} to the protein, the extent of incorporation can still be estimated when the concentration of the protein and the spectral characteristics of the hapten and protein are known. The difference in absorbance between the conjugate and the starting protein is proportional to

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Table 7 Conjugation of an amino-containing hapten to protein using the diazotization method

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- DMF (>99%, from Aldrich)
- Sodium nitrite (0.2 M) in water
- Phosphate buffer (0.1 M, pH 8.8): prepared from KH_2PO_4 (1.40 g), Na_2HPO_4 (2.04 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.10 mmol) in 4 drops of ethanol and treat with 1 mL of 1 N HCl
2. Stir the solution in an ice-bath while adding 0.5 mL of 0.20 M sodium nitrite
3. Add 0.4 mL of DMF dropwise to give a homogeneous solution
4. Dissolve 45 mg of BSA in 5 mL of 0.2 M borate buffer (pH 8.8) and 1.5 mL of DMF
5. Add the activated hapten solution dropwise to the stirred protein solution. Stir in an ice-bath for 45 min
6. Purify conjugate by gel filtration, dialysis or ethanol precipitation

the amount of hapten conjugated.[41] Hapten density can also be determined indirectly by measuring the difference in free amino groups between conjugated and unconjugated protein using trinitrobenzenesulfonic acid.[58] These methods are at best rough estimates because the process of conjugation usually alters the apparent number of amine or sulfhydryl groups on the protein. Careful titration of reactive groups on very large proteins is particularly difficult.

Alternatively, competitive ELISA can be used to estimate the hapten density if an antibody that specifically recognizes the hapten is available.[59] At first observation this approach seems circular because the immunoassay developed is used to determine hapten density on proteins used for immunization. However, if a small molecule mimic of the protein conjugate is used as a standard, the method can be accurate. For example, a hapten containing a carboxylic acid can be coupled to phenethylamine or tyramine, its structure confirmed and the material used to generate a calibratron curve to estimate hapten density.

Advanced mass spectrometry (MS) techniques offer a new way of determining the hapten density of protein conjugates. For example, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) detects covalently bound haptens.[60] Increasingly powerful instruments allow higher resolution of conjugates. However, large proteins cannot be analyzed by MS. Protein heterogeneity and some post-translational modifications, particularly glycosylation, will obscure the results and lower resolution instruments cannot distinguish among desired conjugates and unwanted reaction by-products. It is possible, however, to measure hapten density on small peptides unequivocally by MS techniques and extrapolate to proteins such as KLH and thyroglobulin that are too large and/or heterologous for MS analysis.

Hapten density, and also the common positions where haptens are bound, can also be estimated by cyanogen bromide or enzymatic cleavage of the protein and either MALDI-MS or separation of the components by reversed-phase ion-pair chromatography and electrospray or electrospray time-of-flight (TOF) analysis.

Conjugates with a broad range of hapten/protein or hapten/enzyme ratios of about 1–30 have been used successfully to elicit antibody production or as enzyme tracers.[29,61,62] The optimum hapten ratio may depend on the study objectives, the nature of the antigen, immunization protocol, etc. A general rule of thumb is to target high hapten ratios for immunogens and low hapten ratios for coating antigens or enzyme tracers. For immunogens, a high hapten ratio implies greater exposure of the immune system to the hapten; for coating antigens or enzyme tracers, a lower hapten density implies fewer haptens to compete with the analyte in the assay. Optimum hapten density is often determined empirically with checkerboard titration procedures. Such procedures are very rapid and are normally adequate to optimize ELISAs without knowing the exact hapten density. With the development of more sophisticated biosensors, the determination of exact hapten densities may become increasingly important.

2.5.6 Antibody production

Essentially any vertebrate can be used as a source of antibodies. Rabbits are easy to care for, and produce a moderate amount of serum, often with high antibody titers.

Goats or sheep also produce high-quality antiserum in larger amounts. Antibodies derived from serum consist of a population of antibodies that recognize a variety of antigenic determinants with varying degrees of specificity and affinity and are thus termed polyclonal. Although two antisera are rarely identical, even if they come from the same rabbit at different times, it is simple to evaluate each antiserum for specificity and affinity.

In contrast, monoclonal antibodies are obtained from a murine cell line ultimately traceable to a single cloned cell. If carefully screened and selected, the monoclonal antibody will recognize a single antigenic determinant with constant affinity and specificity. The hybrid cell line comes ultimately from spleen lymphocytes (from a previously immunized animal) that have been fused to an immortal myeloma cell line. This fusion ensures that the cell line will continue to produce the selected antibody while it grows and replicates. Although it is attractive to have a permanent supply of antibody with constant specificity and affinity, these cell lines may contain an unstable chromosome complement and their immortality depends upon proper storage and maintenance. The advantages, disadvantages, and production of monoclonal antibodies have been discussed.[63–65]

Immunization procedures and schedules vary depending on the laboratory.[66,67] Usually an initial series of injections is followed by booster injections some weeks later. Animals are generally bled 7–14 days after each booster injection and the characteristics of the serum determined. Serum may be collected or pooled following numerous booster injections and(or) the animal may be exsanguinated.

For long-term storage, antibodies are best stored frozen either in solution or as a lyophilized powder. Similarly to most biological materials, repeated freeze–thaw cycles are detrimental to antibodies, and hence antibodies should be stored in clearly labeled aliquots. A single vial may be used for a set of experiments extending over several months. Antibodies can be kept in solution containing 0.1% sodium azide (to prevent growth of microorganisms) in a refrigerator for up to a year. Solutions can also go through freeze–thaw cycles several times without alarming loss of activity. Although antibodies are relatively hardy proteins, the concentration should be kept above 1 mg mL^{-1} during storage, solutions should be frozen quickly in liquid nitrogen before placing in a standard freezer, and for long-term storage antibodies should be lyophilized and the container sealed under dry nitrogen.

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Building on the monoclonal antibody technology and the advent of molecular biology techniques, it is now possible to isolate antibodies from combinatorial libraries and express them in a variety of expression systems. Efficient systems for the cloning and expression of antibody genes in bacteria were developed in the late 1980s.[68] The discovery of PCR simplified the cloning of monoclonal antibody genes from mouse monoclonal cell lines. These functional recombinant antibody fragments could be expressed in bacteria for use.[69] To take advantage of recombinant technology, efficient, large-scale screening techniques must be used. A variety of techniques have been reviewed by Maynard and Georgiou.[70] The ability to engineer antibodies for therapeutic uses, such as neutralizing toxins (antivenoms), cancer therapy and imaging of tumors, is attractive. For environmental residue analysis, the most likely use of recombinant antibodies is as detector molecules in biosensors, where engineering could provide useful surface linkage chemistry, unique labels or improved robustness of the sensor. A few recombinant

antibodies for pesticides have been developed and at least one applied to a sensor format.[71–75]

2.5.7 Assay optimization

Assay optimization involves determining the optimum coating antigen/hapten–enzyme conjugate and anti-pesticide antiserum concentrations using a checkerboard titration. Using a 96-well plate, the coating antigen concentration is varied by row and the antibody concentration is varied by column so that each well has a different combination of antigen and antibody concentrations. By plotting the resulting absorbance values versus either reagent concentration an estimate can be made of the concentrations that will yield a reasonable signal and at which the system is not saturated.[76]

Using the optimum reagent concentrations, the assay is tested for inhibition by the target analyte. If a useable IC_{50} is obtained, then further optimization is conducted. This second stage of optimization includes determining the optimum assay temperature and incubation times and the effect of potential interferences (e.g., solvent, salt, pH, matrix). When evaluating immunoassays, it is important to remember that the law of mass action applies and interferences affect the equilibrium condition. For example, assays are conducted with reagents that have been equilibrated to room temperature. If room temperature is not constant (within 3–5 °C), then assays should be conducted using a forced-air incubator. Shaking the plate periodically during incubation may improve precision because reactions occur at the surface of the microtiter plate, causing a localized concentration of reactants. For immunoassays utilizing 30-min or longer incubation periods, the reactants have likely come nearly to equilibrium, and precise timing of the incubation period is less critical than for nonequilibrium immunoassays. Each of these variables should be evaluated and controlled if necessary in order to improve the precision of the measurements.

2.5.8 Validation

Consistent with other analytical methods, immunoassays must be validated to ensure that assay results are accurate. Initial validation involves an evaluation of the sensitivity and specificity of the immunoassay, while later validation includes comparison with a reference method. Because a goal of immunoassays is to minimize sample preparation, validation also includes testing the effects of sample matrices and(or) sample cleanup methods on results. The final steps in validation involve testing a limited number of samples containing incurred residues to determine if the method provides reliable data.

Structurally related compounds may cross-react with the antibody, yielding inaccurate results. In screening for the herbicide alachlor in well water by immunoassay, a number of false positives were reported when compared with gas chromatography (GC) analysis. A metabolite of alachlor was found to be present in the samples and it was subsequently determined that the cross-reactivity by this metabolite accounted for the false-positive results.[77] On the other hand, cross-reactivity by certain structural analogs may not be an issue. For example, in an assay for the herbicide atrazine, cross-reactivity by propazine is 196%;[78] because of atrazine and propazine field use

patterns, they are not usually found together. Conversely, this assay also cross-reacts with simazine by 30% and simazine is expected to be present. Hence, if the sample is positive and the presence of simazine is expected, another method of analysis would be necessary to determine the relative contribution of each triazine.

The second phase of validation involves comparing the immunoassay with an established method with a known accuracy using an identical same sample set. For most pesticides, reference methods are based on gas chromatography/mass spectrometry (GC/MS) or high-performance liquid chromatography (HPLC). When comparing two methods, it is important to be aware of the strengths and weaknesses of each. For example, many pesticide immunoassays require minimum sample cleanup before analysis, relative to the corresponding GC/MS or HPLC methods. Thus, immunoassay data may reflect higher values if there are losses occurring during further sample workup for GC/MS. On the other hand, the immunoassay data may be higher because a cross-reacting species is present that the GC/MS differentiates by chromatography. Comparison of immunoassay results with results obtained from a validated method will determine if the immunoassay is accurate.

For pesticide residue immunoassays, matrices may include surface or groundwater, soil, sediment and plant or animal tissue or fluids. Aqueous samples may not require preparation prior to analysis, other than concentration. For other matrices, extractions or other cleanup steps are needed and these steps require the integration of the extracting solvent with the immunoassay.[79] When solvent extraction is required, solvent effects on the assay are determined during assay optimization. Another option is to extract in the desired solvent, then conduct a solvent exchange into a more miscible solvent. Immunoassays perform best with water-miscible solvents when solvent concentrations are below 20%. Our experience has been that nearly every matrix requires a complete validation. Various soil types and even urine samples from different animals within a species may cause enough variation that validation in only a few samples is not sufficient.

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Matrix effects are determined by running calibration curves in various dilutions of matrix and comparing the results with those for corresponding calibration curves run in buffer. Overlapping curves indicate no effect of matrix. Parallel curves are an indication that a matrix interference is binding the antibody in the same manner as the analyte. Nonparallel curves are indicative of nonspecific matrix interferences. Grotjan and Keel[21] described parallelism tests, similarity of curves and the corresponding statistics. A second test for matrix effects is to analyze a sample before and after a known amount of analyte has been added (test of additivity). If the values for the 'before' and 'after' samples are not additive, a matrix effect is presumed. If matrix effects are present, then adjustment of the immunoassay method, such as running the calibration curve in the matrix or further sample preparation, is necessary.

2.5.9 Quality control (QC) and troubleshooting

Unlike GC/MS methods, internal standards are not appropriate for immunoassays. Internal standards that would react with the antibody but would not interfere with the assay are nonexistent. In the place of internal standards, external QC must be maintained.

One strategy is to use appropriately stored batch QC samples that are analyzed with each assay because intra- and interassay variability are easily tracked. Various types of QC samples can be employed to demonstrate the performance of the assay. A blank sample such as an empty well or buffered solution can indicate any background response that can be subtracted from the sample and standard responses. A negative control sample (i.e., matrix extract solution known to contain no analyte) can reveal whether a nonspecific response or matrix effect is occurring. A positive control or matrix extract fortified with a known amount of the analyte can determine accuracy. Precision can be determined using standards and samples run in replicate. Blanks, negative controls, positive controls, fortified sample extracts standardized reference material extracts and replicates are typically run on each microplate to control for plate-to-plate variation.[80] Recording assay accuracy and precision and maximum (no analyte present) and minimum (completely inhibited) absorbances over time will provide a warning of deteriorating assays.[81,82]

If an assay does not meet performance criteria, there are a variety of corrective measures (Table 8). The most frequent immunoassay performance problem is a high coefficient of variation for replicates or spurious color development. Plate washing and pipetting techniques are the greatest sources of this error.[76,83] A decrease in the maximum absorbance can be attributed to loss of enzyme activity or hapten conjugate degradation. To check enzyme activity, dilute the enzyme–conjugate about 2–5 times greater than normal for the assay. For example, if the method calls for a 1:2500 dilution of the enzyme label, then make dilutions of 1:5000 to 1:10 000, or greater. Add the substrate solution to the enzyme dilution and incubate for the time indicated in the method. Color development should be similar to that obtained in the assay when it is performing according to specifications. If the color development is lower, the enzyme label reagent should be replaced. Hapten–conjugate degradation can only be remedied by replacing the reagent.

Another important factor for QC is temperature. Reagents should be used at room temperature and plates should be protected from wide fluctuations in temperature while conducting the immunoassay. If an incubator is used or the ambient temperature is high, uneven heating of the wells may occur. Variations in final absorbances may be manifested in what is called an 'edge effect', in which greater variation occurs among the wells on the edges of the plate. Use of a forced-air incubator can reduce this problem. Detailed immunoassay troubleshooting information has been presented by Schneider *et al.*[84]

2.6 Applications

Pesticide immunoassays have been developed for a variety of pesticides and, more recently, GMOs, and have been used for matrices such as surface water, groundwater, runoff water, soil, sediment, crops, milk, meat, eggs, grain, urine and blood.[85–90] Table 9 is a partial list of immunoassays for chemical pesticides developed since 1995 and includes notations on the matrices studied. A fairly comprehensive list of pesticide immunoassays developed prior to 1994 was provided by Gee *et al.*[91]

Table 8 Troubleshooting the optimized immunoassay

Symptom	Cause	Remedy
Poor well to well replication	Poor pipetting technique	Check instrument, practice pipetting, calibrate pipet
	Poor binding plates	Check new lot, change manufacturer
	Coating antigen or antibody is degrading	Use new lot of coating reagent or antibody
	Coated plates stored too long	Discard plates, coat a new set, decrease storage time
	Poor washing	Wash plates more, or more carefully, remake buffer
	Uneven temperature in the wells	Deliver reagents at room temperature, avoid large temperature fluctuations in the room
	Sample carryover	Watch for potential carryover in pipetting and washing steps
Low or no color development	Loss of reagent integrity	Systematically replace or check reagents, including buffers and beginning with the enzyme label
	Incubation temperature too cold	Lengthen incubation time or increase temperature by using a circulating air-temperature controlled incubator
	Sample matrix effect	Dilute matrix if possible, check pH of matrix, increase the ionic strength of the buffer, re-evaluate matrix
Color development too high	Incubation too long or temperature too high	Decrease incubation time or temperature
	Matrix effect	Dilute matrix or re-evaluate matrix effects
Change in calibration curve parameters	Degradation of reagents	Systematically check or replace reagents, including buffers

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2.6.1 Human exposure monitoring

The immunoassay is one of the most promising methods for the rapid monitoring and assessment of human exposure. The great specificity and sensitivity of immunoassays allow their use for monitoring pesticide exposure levels by determining parent compound, key metabolites[92] or their conjugates in human urine, blood,[93] and(or) saliva.[94] Recently, several immunoassays have been developed to assess human exposure to alachlor,[95,96] atrazine,[97,98] metolachlor,[99] and pyrethroids.[100] In the case of the herbicide atrazine, the mercapturic acid conjugate excreted in human urine[101] is a specific biomarker for exposure. A sensitive immunoassay has been developed for this metabolite[97] that can be detected at 0.1 μg L^{-1} in urine. The great advantage of the immunoassay over chromatographic methods is high throughput, which is

Table 9 Immunoassays developed since 1995

Class	Name, matrix	Reference
Herbicide	Chlorpropham, food	139
	Isoproturon, water	140
	Metsulfuron-methyl, water	141, 142
	Bensulfuron-methyl, water	143
	Chlorsulfuron	144
	Fluometuron, soil	145
	Trifluralin, soil, water, food	146, 147
	Cyclohexanedione	148, 149
	Triazines, water, food	19, 150, 151
	Dichlobenil	152
	Propanil, water	153
	Dichlorprop methyl ester	154
	Hexazinone, water	155
	Fluroxypyr, triclopyr, soil	156
Insect growth regulator	Fenoxycarb	157, 158
	Flufenoxuron, soil, water	159
Insecticide	Hexachlorocyclohexane, water, soil	160
	Azinphos-methyl, water	161
	Carbofuran, food	162–164
	Chlorpyrifos, water	165, 166
	Chlorpyrifos-ethyl	74
	Pymetrozine, plants	167
	Azinophos-methyl, water	161, 168
	Pyrethroids	37, 39, 169
	Allethrin	170
	Esfenvalerate, water	36
	Flucythrinate, soil, water, food	171
	Permethrin, air, water	35, 172
	Organophosphates	112, 173, 174
	Fenitrothion, food, water	175, 176
	DDT, soil, food	177–179
	Etofenprox	180
	Phosalone	181
	Spinosyn A, water	182
	Spinosad, food, water, sediment	89, 183
	Imidacloprid, water, food	13, 175, 184
	Acetamiprid, water, food	175
	Azadirachtin, food, formulations	185
	Oxamyl, food	186
	Propoxur	187
Fungicide	Myclobutanil, soil, water, food	188
	Procymidone, food	189
	Benalaxyl, food, water	190
	Thiram, food	191, 192
	Chlorothalonil, water, plant residues, food	193–195
	Tebuconazole, food	196, 197
	Thiabendazole, food	198–200
	Imazalil, food	201
	Tetraconazole	197, 202
	Myclobutanil, water, soil, food	188, 202
	Hexaconazole, formulations	203
	Didecyldimethylammonium chloride	204
	Methyl 2-benzimidazolecarbamate, soil, food	205, 206
	Captan, food, water	207

particularly suitable for screening large numbers of samples generated during human exposure studies.

2.6.2 Immunoassay in agricultural biotechnology

Agricultural biotechnology providers include agricultural biotechnology companies, seed companies, food companies and other research organizations. Technology providers use qualitative, quantitative and threshold immunoassays during all stages of the research and development of biotech crops, the choice depending on the specific application. Immunoassays are used for gene discovery, event selection, screening, transformant identification, line selection, plant breeding and seed quality control. Agricultural biotechnology companies also use immunoassays for product support, product stewardship and intellectual property protection.

Technology providers use quantitative immunoassays to determine expression data of field material for regulatory submissions. Regulatory authorities require that expression levels of introduced proteins in various plant parts be determined by quantitative, validated methods. Immunoassays are also used to generate product characterization data, to assess food, feed and environmental characteristics, to calculate concentrations for toxicology studies and to obtain tolerance exemption or establish tolerances for pesticidal proteins.

Immunoassays are also useful in the food handling and distribution system. Threshold assays are most commonly used to test agricultural commodities entering the food distribution channel to ensure compliance with relevant labeling regulations.[102] Immunoassays can be applied to raw, fresh and or lightly processed foods. The protein analyte can be denatured during processes such as heating. This creates potential difficulties in the analysis of heavily processed finished food products.

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2.6.3 Flow injection immunoassay (FIIA)

In FIIA, antibodies are immobilized to form an affinity column and analyte is pumped over the column. The loading of the antibodies with analyte is followed by pumping over the column enzyme tracers that compete with the pesticide for the limited binding sites of the antibodies. Generally, the indirect format produces a result inversely proportional to the pesticide concentration. FIIA can be used with electrochemical, spectrophotometric, fluorimetric and chemiluminescence detection methods. Conventional UV visible spectrophotometry is also suitable for the FIIA detection of bioligand interactions.[103] FIIA has been used for the detection of diuron and atrazine in water.[104] The method was developed as a cost-effective screen for determining compliance with the European drinking water directive. One analysis for either atrazine or diuron, including column regeneration, took about 50 min using the system that is shown schematically in Figure 18. The column material was regenerated up to 1600 times over a 2.5 month period. FIIA is a powerful analytical tool for semi-continuous, high sample throughput applications and may serve as an alternative or complementary technique to solid-phase immunoassay by providing real-time monitoring data.[105] In addition, the continuous flow system is easier to automate than assays using tubes or microplates. More rapid results and sensitive detection will be possible by miniaturizing the column and fluid handling and

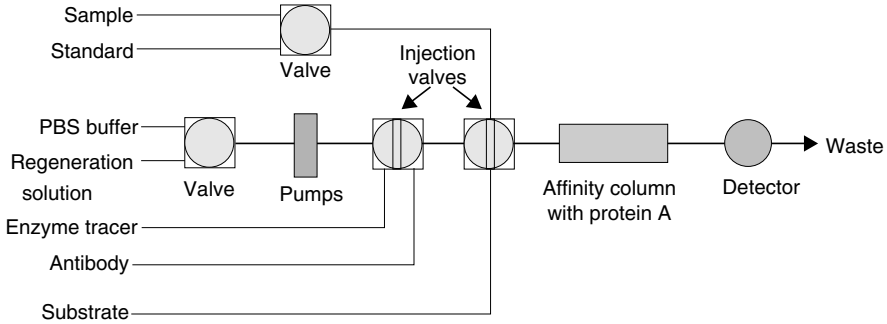


Figure 18 Flow chart of the automated on-line flow injection immunoassay (FIIA). Six steps are involved in each cycle: (1) addition of antibody and incubation; (2) addition of analyte (or standard) and incubation; (3) addition of enzyme–tracer and incubation; (4) addition of substrate and incubation; (5) downstream measurement of fluorescence; (6) regeneration of affinity column

with the development of sensors that can detect antibody–antigen binding events directly.

2.6.4 Multi-analyte analysis

Immunoassays traditionally have been used as a single-analyte method, and this is often a limitation of the technology. However, several approaches are possible to overcome this limitation. A simple approach is to have highly selective assays in different wells of a single microtiter plate, as was demonstrated for the sulfonylureas.[106] A more elegant approach than using a microtiter plate is to use a compact disk (CD)-based microarray system.[107] A microdot system was developed that utilized inkjet technology to 'print' microdots on a CD. The CD was the solid phase for immunoassay, and laser optics were used to detect the near-infrared fluorescent label. The advantage of the CD system is the ability both to conduct assays and to record and/or read data from the same CD. Since the surface of a single CD can hold thousands of dots, thousands of analyses can be made on a single sample simultaneously. Such high-density analyses could lead to environmental tasters where arrays of immunosensors are placed on chips[108,109] or high-density plates. Because the CD format has the potential for high-density analyses, there will be the opportunity for easily generating multiple replicates of the same sample, including more calibration standards, thus improving data quality.

The development of class-selective antibodies is another approach to multi-analyte analysis. The analyst may design haptens that will generate antibodies that recognize an epitope common to several compounds, as explained above for the analysis of pyrethroids by measuring PBA. Other examples of class-selective immunoassays that have been developed are mercapturates,[110] glucuronides,[111] pyrethroids,[37,39] organophosphate insecticides,[112] and benzoylphenylurea insecticides.[113]

Rather than have one antibody that can detect a class, a third approach is to analyze a sample using multiple immunoassays, each with a known cross-reactivity spectrum, and determine the concentration of the analytes and confidence limits mathematically.[114–116] A drawback to using class-selective assays or assays with known cross-reactivity is that for a given antibody, the sensitivity for each analyte

will vary, and the sensitivity for some analytes may not be sufficient, hence selection of well-characterized antibodies will be a critical step.

2.6.5 Future prospects

Immunoassays designed for environmental applications are mostly sold as some variation of the ELISA format. ELISA-like formats dominate the field because they are inexpensive and because they provide high sensitivity and precision without requiring complex instrumentation. The basic ELISA format supports both field and laboratory-based applications but is limited by multiple steps and inadequate sensitivity for some applications, excessive variability and sometimes long analysis times. Some of the other formats discussed in this article may replace the ELISA for selected applications; however, because many laboratories are familiar with the ELISA technology, there will be a significant delay before alternative formats are widely accepted.

In the near term, to improve throughput, the 96-well ELISA is likely to be replaced by higher density arrays. For example, plates, readers and robotic systems are being developed for high-throughput screening in the pharmaceutical industry in 384-, 768-, and 1536-well formats. Other high-throughput formats will utilize inkjet printing technology on CD surfaces or FIIA-like systems, which offer advantages for sequential analysis as discussed above. Biosensor technology will also likely be integrated with ELISAs to generate improved formats.

It is critical to keep in mind that existing reagents can be used for multiple formats. For example, polyclonal antibodies dominate the environmental field because they generally provide greater sensitivity and specificity for small molecules at a much lower cost than do monoclonal or recombinant antibodies. With some biosensors monoclonal or engineered antibodies or recombinant binding proteins may offer advantages.

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3 PCR for products of agricultural biotechnology

The recent introduction of genetically modified crops has changed both the agriculture and food industries. United States Department of Agriculture (USDA) surveys report that 25% of corn, 61% of cotton and 54% of soybean acreage grown in the USA in 2000 were genetically modified.[117]

Agricultural biotechnology involves inserting a novel gene [deoxyribonucleic acid (DNA) sequence] into plants or animals using recombinant DNA techniques. These techniques even allow the transfer of DNA from a donor organism to a recipient organism that is not genetically related, a feat not possible using conventional breeding techniques. The novel DNA codes for the expression of a specific protein that confers a new trait or characteristic to the plant or animal. Most traits are described as either input or output traits. Input traits are useful for crop production and include commercial biotech crops that contain herbicide tolerance or resistance to insect pests or diseases. Output traits offer valuable quality enhancements such as improved nutritional value or improved handling or processing characteristics.

Since the commercial introduction of biotech crops, a need has emerged for analytical methods capable of detecting the novel DNA sequences introduced into the plant genome and also methods for detecting the protein products expressed by the

plant. PCR is a powerful tool for the amplification and detection of defined DNA sequences. This section describes the basic principles of agricultural biotechnology and covers principles of both conventional and real-time PCR for DNA analysis. Examples of how these techniques are currently used for analytical testing of raw agricultural commodities and finished food are presented.

3.1 Basic principles of agricultural biotechnology

Within the nuclei of plant cells, chromosomal DNA provides instructions for the cells to replicate themselves and to carry out vital functions. Individual, unique DNA sequences (genes) code for the production of individual, unique proteins. With the tools of modern biotechnology, it is possible to introduce novel DNA sequences that instruct plant cells to synthesize or over-express proteins that confer new traits to the plant. It is also possible to 'down-regulate' or turn off a native gene, thereby suppressing or eliminating the synthesis of a native protein, which can also produce a new trait. Plants that have been transformed in these ways have been called transgenic, genetically modified (GM), genetically engineered (GE), biotech plants and(or) genetically modified organisms (GMOs).

There are several methods that can be used to introduce foreign genes into plant cells, a process called, in general, transformation. Among the most common plant transformation methods are biolistics and exposure to *Agrobacterium tumefaciens.*

Biolistics involves bombarding plant cells with tiny (4-μm) microprojectiles made of gold or tungsten. These microprojectiles are coated with DNA and are propelled at high velocity from a particle gun or 'gene gun' into plant tissue or cells. In this method, the projectile penetrates the cell wall and carries the transgene into the cell nucleus.

A. tumefaciens is naturally able to transform a wide variety of plant species. Mature differentiated plant tissue (an explant) is exposed to *A. tumefaciens* bacteria harboring a 'foreign' gene. The bacterial infection results in foreign DNA from the bacterium being transferred into the genome of the host plant, and results in a crown gall tumor. This naturally occurring process can easily be exploited to produce a transgenic plant.

Plasmids are often used as vectors to transfer DNA into plant cells. In particular, the tumor-inducing (Ti) plasmid of *A. tumefaciens* is a common vector. Plasmids are extrachromosomal, autonomously replicating, circular double strands of DNA that can occur in high copy number in a bacterial cell. It is possible to construct a recombinant Ti plasmid by inserting an effect gene, regulatory sequences (such as transcriptional promoters and terminators), along with a selectable marker gene (such as antibiotic or herbicide resistance) into the circular plasmid.

After the recombinant plasmid has been constructed using *in vitro* methods, leaf disks or protoplasts are infected with recombinant *A. tumefaciens* cells. The infection process incorporates the foreign gene and other genetic elements into the host-plant genome. The host cells are then regenerated from undifferentiated callus tissue into a transgenic plant in tissue culture. Only some of the cells receive the gene of interest, so it is necessary for explants to be grown up in a selective medium.[118]

In order for any gene to synthesize a protein, it must contain certain genetic elements such as promoter and terminator sequences. These regulatory regions signal where the DNA sequence that encodes a product (i.e., a gene) begins and ends. The recombinant

DNA construct will often contain an effect gene and a selectable marker gene (such as antibiotic or herbicide resistance), both of which are bracketed by promoter and terminator sequences. A plasmid vector carries this cassette of genetic information into the plant genome by one of the above methods.

Multiple or 'stacked' traits are sometimes introduced into a single plant. These could include resistance to multiple viruses, fungal resistance, etc. Each of these stacked-trait genes usually has an associated promoter and terminator sequence. Obtaining information about particular gene constructs, including marker and regulatory sequences, is vital for PCR testing to detect GMOs in a crop or food sample. The required sequence information can be inferred by restriction mapping of the recombinant plasmid or, more commonly, by DNA sequencing.

GMO screening often relies on the common genetic elements that are present in many commercial GMOs. Many genetically modified plants use common regulatory sequences and/or marker genes, which makes it possible to simultaneously screen for many GMOs by detecting these sequences. The cauliflower mosaic virus (CaMV) 35S-promoter and the *A. tumefaciens nos*-terminator are examples of two DNA sequences that are present in many commercial GMOs.

A positive result for one of these sequences does not necessarily indicate that the test sample contains GM material. Since the 35S-promoter comes from a virus that infects cauliflower, positive results from plants that belong to the genus *Brassica* would need to be carefully evaluated. Likewise, the *nos*-terminator originated in *A. tumefaciens* and this soil bacterium has a broad spectrum of potential hosts. *Nos*-positive results must be confirmed to rule out bacterial contamination. Testing for these common genetic elements only serves as a GMO screening; it is necessary to apply a specific test to determine which GMO is present in the sample. The following list gives some genetic elements that are commonly detected in GMO screening tests:

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- *CaMV 35S* promoter: a promoter sequence from the CaMV
- *nos* terminator: nopaline synthase, a terminator sequence from *A. tumefaciens*
- *bar* gene: a herbicide resistance selectable marker from *Streptomyces hygroscopicus* that encodes phosphinothricin acetyltransferase
- *pat* gene: phosphinothricin acetyltransferase, a herbicide resistance selectable marker
- *npt II*: neomycin phosphotransferase, an antibiotic resistance selectable marker.[119]

For PCR analysis of a specific GMO, it is necessary to have sequence information about the gene construct, so primers can be designed to be specific to a gene or to a sequence that bridges genetic elements of the specific construct. An example is the specific test for the genetic modification in Roundup Ready soybeans. The target sequence is the transition that links the transit peptide gene from petunia to the 35S promoter region. This transition DNA sequence is specific to Roundup Ready soybeans.

Table 10 lists United States Food and Drug Administration (FDA) submissions in 2000 for commercial GMOs, including the food, gene, source and intended effect.[120]

Table 10 Commercial GMOs

Food[a] Company/year	Gene, gene product, or gene fragment	Source	Intended effect
Corn* DowAgro/2000	Cry1F protein, phosphinothricin acetyltransferase (PAT)	*Bacillus thuringiensis*, *Streptomyces viridochromogenes*	Resistance to certain lepidopteran insects; tolerance to the herbicide glufosinate
Corn Monsanto/2000	5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS)	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Corn Aventis/1999	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Rice Aventis/1999	PAT	*Streptomyces hygroscopicus*	Tolerance to the herbicide glufosinate
Canola Rhone-Poulenc/1999	Nitrilase	*Klebsiella ozaenae* subsp. *ozaenae*	Tolerance to the herbicide bromoxynil
Cantaloupe Agritope/1999	*S*-Adenosylmethionine hydrolase	*Escherichia coli* bacteriophage T3	Delayed fruit ripening due to reduced ethylene synthesis
Canola BASF/1997	Phytase	*Aspergillus niger* van Tieghem	Degradation of phytate in animal feed
Canola AgrEvo/1998	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Canola AgrEvo/1998	Barstar, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Fertility restorer, tolerance to glufosinate
Sugar beet Monsanto and Novartis/1998	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Soybean AgrEvo/1998	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Tomato* Calgene/1997	CryIAc protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to certain lepidopteran insects
Corn Monsanto/1997	Modified EPSPS	Corn	Tolerance to the herbicide glyphosate
Flax University of Saskatchewan/1997	Acetolactate synthase (csr-1)	*Arabidopsis*	Tolerance to the herbicide sulfonylurea
Potato* Monsanto/1997	CryIIIA, PVY coat protein	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt), potato virus Y (PVY)	Resistance to Colorado potato beetle and PVY
Potato* Monsanto/1997	CryIIIA, PLRV replicase	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt), potato leafroll virus (PLRV)	Resistance to Colorado potato beetle and PLRV
Cotton* Calgene/1997	Nitrilase, Cry1Ac protein	*Klebsiella pneumoniae* subsp. ozaene, *Bacillus thuringiensis* var. *kurstaki* (Btk)	Tolerance to the herbicide bromoxynil, resistance to certain lepidopteran insects
Corn* AgrEvo/1998	Cry9C protein, PAT	*Bacillus thuringiensis* subsp. *tolworthi* (Bt), *Streptomyces hygroscopicus*	Resistance to several lepidopteran insects, tolerance to the herbicide glufosinate

Sugar beet AgrEvo/1998	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Corn Pioneer Hi-Bred/1998	DNA adenine methylase (DAM), PAT	*Escherichia coli*, *Streptomyces viridochromogenes*	Male sterility, tolerance to glufosinate
Canola AgrEvo/1997	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Radicchio Bejo Zaden/1997	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Squash* Seminis Vegetable Seeds/1997	Coat proteins from CMV, ZYMV and WMV2	Cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus 2 (WMV2)	Resistance to the viruses CMV, ZYMV and WMV2
Papaya* University of Hawaii/1997	PRV coat protein	Papaya ringspot virus (PRSV)	Resistance to PRSV
Corn* Dekalb Genetics/1996	CryIAc	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Soybean DuPont/1996	GmFad2-1 gene to suppress endogenous GmFad2-1 gene, which encodes delta-12 desaturase	Soybean	High oleic acid soybean oil
Corn* Monsanto/1996	CryIAb protein, EPSPS, glyphosate oxidoreductase	*Bacillus thuringiensis* subsp. *kurstaki* (Btk), *Agrobacterium* sp. strain CP4, *Ochrobactrum anthropi*	Resistance to European corn borer, tolerance to the herbicide glyphosate
Corn Monsanto/1996	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Potato* Monsanto/1996	CryIIIA protein	*Bacillus thuringiensis* var. *tenebrionis* (Btt)	Resistance to Colorado potato beetle
Oilseed rape Plant Genetic Systems/1995	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Oilseed rape (Canola) Plant Genetic Systems/1995	Barstar, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Fertility restorer, tolerance to glufosinate
Oilseed rape Plant Genetic Systems, America/1996	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Cotton Dupont/1996	Acetolactate synthase (ALS)	*Nicotiana tabacum* cv. *Xanthi* (tobacco)	Tolerance to the herbicide sulfonylurea
Corn Dekalb Genetics/1995	PAT	*Streptomyces hygroscopicus*	Tolerance to the herbicide glufosinate
Corn* Monsanto/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Corn* Northrup King/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer

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Table 10—*Continued*

Food[a] Company/year	Gene, gene product, or gene fragment	Source	Intended effect
Tomato Agritrope/1996	*S*-Adenosylmethionine hydrolase	*Escherichia coli* bacteriophage T3	Delayed fruit ripening due to reduced ethylene synthesis
Corn AgrEvo/1995	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Cotton Monsanto/1995	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Oilseed rape (Canola) Calgene/1992	12:0 Acyl carrier protein thioesterase	*Umbellularia californica* (California Bay)	High-laurate canola oil
Corn* Ciba-Geigy/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstak*i (Btk)	Resistance to European corn borer
Oilseed rape (Canola) AgrEvo/1995	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Oilseed rape (Canola) Monsanto/1995	EPSPS, glyphosate oxidoreductase (GOX)	*Agrobacterium* sp. strain CP4, *Achromobacte*r sp. strain LBAA	Tolerance to the herbicide glyphosate
Cotton* Monsanto/1994	CryIAc protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to cotton bollworm, pink bollworm and tobacco budworm
Tomato DNA Plant Technology/1994	A fragment of the gene encoding aminocyclopropanecarboxylic acid synthase (ACCS) to suppress the endogenous ACCS enzyme	Tomato	Delayed ripening due to reduced ethylene synthesis
Squash* Asgrow/1994	ZYMV and WMV2 coat proteins	ZYMV and WMV2	Resistance to ZYMV and WMV2
Potato* Monsanto/1994	CryIIIA protein	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt)	Resistance to Colorado potato beetle
Cotton Calgene/1994	Nitrilase	*Klebsiella ozaenae*	Tolerance to the herbicide bromoxynil
Tomato Zeneca/1994	A fragment of the polygalacturonase (PG) gene to suppress the endogenous PG enzyme	Tomato	Delayed softening due to reduced pectin degradation
Tomato Monsanto/1994	1-Aminocyclopropane-1-carboxylic acid deaminase (ACCD)	*Pseudomonas chloraphis*	Delayed softening due to reduced ethylene synthesis
Soybean Monsanto/1994	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Tomato Calgene/1991	Antisense PG gene to suppress the endogenous PG enzyme	Tomato	Delayed softening due to reduced pectin degradation

[a] An asterisk indicates that the modified plant produces a pesticidal substance that is regulated by the United States Environmental Protection Agency (USEPA).

3.2 Basic principles of the PCR

DNA is the molecule that encodes genetic information. DNA is a double-stranded molecule with two sugar–phosphate backbones held together in the shape of a double helix by weak hydrogen bonds between pairs of complementary nitrogenous bases. The four nucleotides found in DNA contain the nitrogenous bases adenine (A), guanine (G), cytosine (C) and thymine (T). A base sequence is the order of nucleotide bases in a DNA molecule. In nature, base pairs (bp) form only between A and T and between G and C; hence the base sequence of each single strand can be deduced from that of its complementary sequence.

The PCR is a method for amplifying a DNA base sequence in vitro using a heat-stable DNA polymerase and two primers, complementary to short sequences flanking the target sequence to be amplified. A primer is a short nucleotide chain, about 20 bp in length, which anneals to its complementary sequence in single-stranded DNA. DNA polymerase, an enzyme that aids in DNA replication, adds new deoxyribonucleotides to the extensible (3′) end of the primer, thereby producing a copy of the original target sequence. Taq polymerase (isolated from a thermophilic bacterium called *Thermus aquaticus*) is the most common heat-stable DNA polymerase used in the PCR.

A PCR cycle involves DNA denaturation, primer annealing and strand elongation. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, the PCR produces rapid and highly specific amplification of the target sequence. Repeated rounds of thermal-cycling result in exponential amplification of the target sequence. Theoretically, 2^n copies of the target can be generated from a single copy in n cycles. There is therefore a theoretical quantitative relationship between number of cycles and starting copy number. This will be covered in more detail in the discussion of real-time PCR.

DropBooks

3.2.1 Isolation and purification of the template DNA

The quantity, quality and purity of the template DNA are important factors in successful PCR amplification. The PCR is an extremely sensitive method capable of detecting trace amounts of DNA in a crop or food sample, so PCR amplification is possible even if a very small quantity of DNA is isolated from the sample. DNA quality can be compromised in highly processed foods such as pastries, breakfast cereals, ready-to-eat meals or food additives owing to the DNA-degrading action of some manufacturing processes. DNA purity is a concern when substances that inhibit the PCR are present in the sample. For example, cocoa-containing foodstuffs contain high levels of plant secondary metabolites, which can lead to irreversible inhibition of the PCR. It is important that these substances are removed prior to PCR amplification. Extraction and purification protocols must be optimized for each type of sample.

Several standard DNA isolation kits are commercially available, including the QIAamp DNA Stool Mini Kit and the DNeasy Plant Mini Kit made by Qiagen. Both of these products are based on silica gel membrane technology and allow for the extraction of total DNA from processed foods and raw foodstuffs, respectively. In

both methods, the cellular components of the samples are first lysed; next the isolated DNA is bound to a membrane gel matrix and washed thoroughly. DNA is then eluted. The DNA Stool Mini Kit includes an extra pre-purification step to remove PCR inhibitors.[121]

Classical approaches to plant DNA isolation aim to produce large quantities of highly purified DNA. However, smaller quantities of crudely extracted plant DNA are often acceptable for PCR analysis. Another efficient method for preparation of plant DNA for PCR is a single-step protocol that involves heating a small amount of plant tissue in a simple solution. Several factors influence nucleic acid release from tissue: salt, EDTA, pH, incubation time and temperature. These factors must be optimized for different sample substrates. EDTA in the sample solution binds the Mg^{2+} cofactor required by the Taq polymerase in the PCR, so the EDTA concentration in the solution, or the Mg^{2+} concentration in the PCR, must be carefully optimized.

An optimized single-step protocol for the extraction of leaf tissue or seed embryos is given here. The template preparation solution (TPS) contains:

100 mM Tris–HCl, pH 9.5
1 M KCl
10 mM EDTA

1. To a sterile 1.7-mL microcentrifuge tube containing 20 μL of TPS, add a maximum of a 2-mm^2 piece of leaf or 0.5-mg piece of embryo and incubate at 95 °C for 10 min.
2. Add a 1-μL portion of the supernatant (or dilution thereof, if inhibitors are present) to the 50-μL PCR reaction.

Making sure that the sample size does not exceed the maximum area or weight is important to minimize the amounts of interfering substances that are coextracted. If the leaf sample is larger than 2 mm^2, coextractive substances can inhibit the PCR assay. Regardless of which extraction method is used, it is important that the PCR assay is evaluated for coextractive interferences or inhibitors.[122]

3.2.2 Components of a PCR

The components necessary for a PCR are assembled in what is known as a mastermix. A PCR mastermix contains water, buffer, $MgCl_2$, dNTPs, forward and reverse primers and DNA polymerase (enzyme). After the mastermix has been assembled, template DNA is added.

1. *Water*: The water used in the assay should be deionized, ultrafiltered and sterile.
2. *Buffer*: The PCR buffer is usually provided as a 10-fold solution and is designed to be compatible with the enzyme. Common buffer components are: 500 mM KCl; 100 mM Tris–HCl, pH 9.3; 1–2% Triton X-100; 0.1% Tween.
3. *$MgCl_2$*: 0.5–3.5 mM $MgCl_2$ salt must be added to the assay, as Mg^{2+} is required as a cofactor for the DNA polymerase.
4. *dNTPs*: Deoxynucleoside triphosphates (dATP, dTTP, dCTP, dGTP) are the nucleotide building blocks for the synthesis of new DNA. The dNTPs are sensitive to repeated freeze–thaw cycles and are usually stored in small aliquots (10 mM pH 7.0); concentrations of 20–200 mM are needed in the assay; too high a

concentration can lead to mispriming and misincorporation of nucleotides. All four nucleotides must have the same concentration in the assay.

5. *Primers*: The primers are short (15–30) oligonucleotide sequences designed to base pair or anneal to complementary sequences that flank the DNA target sequence to be amplified. The primers are added at 0.1–1 μM in the assay.
6. *Enzyme*: Taq polymerase (or some other enzyme) adds new deoxyribonucleotides during strand elongation. Taq is added to the assay at 1 unit per 50 μL of reaction mixture.
7. *DNA*: The template DNA is isolated from cells by some sort of extraction procedure. This is usually the last thing added to the reaction before the tube is placed in the thermal cycler.[123]

3.2.3 Contamination control

Because the PCR exponentially copies the target molecule or molecules, amplicon contamination in the laboratory is a serious concern. It is recommended that the mastermix is prepared in an isolated area, such as a PCR station equipped with a UV light. This work area should be exposed to UV radiation after use to destroy any DNA contaminants. The use of dedicated pipets and filtered pipet tips is also recommended. The template DNA should be prepared and added to the reaction in an area that is isolated from the mastermix preparation hood. The thermal cycling and gel electrophoresis should be conducted in a third work area and care should be taken not to introduce amplified PCR products into the mastermix or template preparation work areas.

DropBooks

3.2.4 Thermal cycling

Once the reaction tube has been placed in the thermal cycler, there are normally three steps in a PCR cycle:

1. *Denaturation step*. This step separates the double-stranded DNA into complementary single strands. Also called melting, this usually occurs at a temperature of about 95 °C for 30 s or 97 °C for 15 s.
2. *Annealing step*. The second step is primer annealing, where the forward and reverse primers find their complementary sequences and bind, forming short double-stranded segments. The annealing temperature (T_a) can be estimated from the melting temperature (T_m) by the following equations:

$$T_a = T_m - 5\,^{\circ}\mathrm{C} \tag{1}$$

$$T_m = (A + T) \times 2 + (\mathrm{C} + \mathrm{G}) \times 4 \tag{2}$$

3. *Elongation step.* The third step is strand elongation, where the DNA polymerase synthesizes new DNA strands starting at the primer sequences. Under optimum conditions, approximately 60 bp are synthesized per second. Typically, elongation takes place at about 72 °C.

The number of PCR cycles depends on the number of source molecules. For 10^5 source molecules, 25–30 cycles are required; for 10^4 source molecules, 30–35 cycles; and for 10^3 source molecules, 35–40 cycles. Running more than 40 cycles can cause the formation of unspecific fragments and does not normally yield any more of the target sequence.[123]

3.2.5 Gel electrophoresis

After amplification, it is necessary to visualize the PCR products. Agarose gel electrophoresis is a technique for separating DNA fragments by size. Purified agar (isolated from seaweed) is cast in a horizontal slab. The agarose slab is submerged in a buffer solution and samples are loaded into wells in the gel. An electric current is applied to electrodes at opposite ends of the gel to establish an electrical field in the gel and the buffer. Because the sugar–phosphate DNA backbone is negatively charged, the fragments migrate by size through the pores in the agarose toward the positive electrode. The addition of an intercalating dye such as ethidium bromide causes bands on the gel to fluoresce under UV radiation.

3.2.6 Multiplex PCR

It is possible to amplify and detect multiple DNA sequences in a single reaction tube by using multiple primer pairs, which recognize and bind to the flanking regions of different specific target sequences. Since the PCR products (amplicons) are separated and visualized according to fragment size, it is important to be sure that the fragments produce bands that can be resolved on a gel during electrophoresis. It is also important to design primers that are not likely to compete or bind to each other to form primer dimers.

3.2.7 Results and data interpretation

Smaller nucleic acid fragments migrate more rapidly than larger ones, hence migration distance can be related to fragment size by comparing bands in sample lanes with a molecular marker containing reference DNAs of known lengths run on the same gel. Solutions are loaded into wells at the top of the gel and the migration distance from the well to the band front is related to the size of the DNA fragment.

The gel photograph in Figure 19 shows seven lanes of data. The 100-bp molecular marker was loaded into lane 1. Sample solutions after PCR were loaded into lanes 2–6. These plant samples were assayed to determine transgenic status. In this multiplex PCR assay, three primer sets were used to amplify three target DNA sequences: top band – species-specific endogenous gene; middle band – introduced effect gene (transgene); bottom band – selectable marker gene (transgene).

The presence of the band for the species-specific endogenous gene in all sample lanes demonstrates that the PCR amplification was successful. It is clear that the plant sample in lane 3 is negative for the transgene of interest, because the only band present is the endogenous species-specific gene. It is clear that the plant samples in lanes 2, 4, 5 and 7 are all positive for the transgene of interest because all three of the target sequences are visible on the gel.

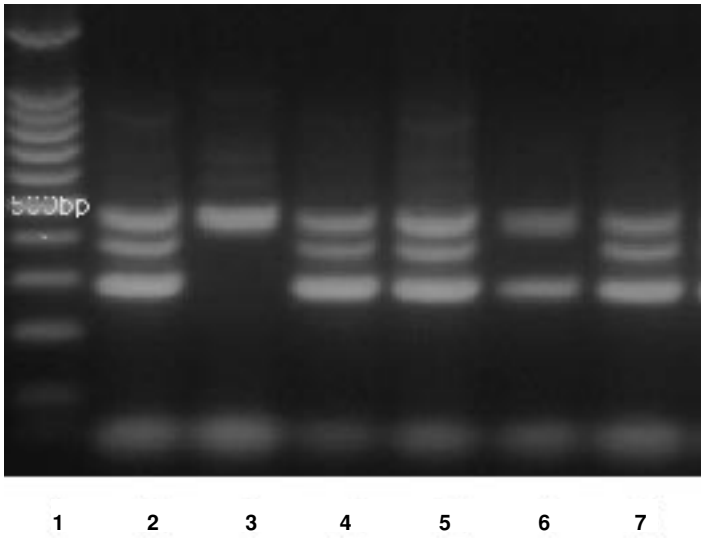


Figure 19 Sample gel of the results of a PCR. Lane 1 is a 100-bp molecular marker; lanes 2–6 are samples. The presence of the top bands (the species-specific endogenous gene) demonstrates that the PCR amplification was successful. Lack of the middle band (the introduced effect gene) and the bottom band (the selectable marker gene) in lane 3 indicates that sample is negative for the effect gene. Presence of all three bands in the remaining lanes indicates the samples are positive for the effect gene

The plant sample in lane 6 is also positive for the transgene of interest. Because the band for the effect gene (middle band) is typically fainter than the band for the selectable marker gene (bottom band), it appears that for lane 6, the PCR product amplification for the effect gene is below the assay detection threshold. Because the selectable marker is clearly present and the PCR amplification worked, lane 6 can be interpreted as a positive result for the transgene of interest.

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3.2.8 PCR controls

There are three types of PCR controls, endogeneous reference genes and negative and positive controls. Primers that amplify a species-specific endogenous reference gene are used as internal controls in the PCR. For example, in a soybean assay, the soy lectin gene may be used as the species-specific reference gene (Table 11).[121] Maize invertase can be used as the endogenous reference gene in corn (Table 12).[121]

Table 11 Primer sequences for PCR analysis of Roundup Ready (RR) Soy

Primer	Sequence (5′–3′)[a]	Length of amplicon (bp)
Lectin	GACGCTATTGTGACCTCCTC	
Lectin	GAAAGTGTCAAGCTTAACAGCCGACG	318
EPSPS RR Soy-specific	TGGCGCCCAAAGCTTGCATGGC	356
EPSPS RR Soy-specific	CCCCAAGTTCCTAAATCTTCAAGT	

[a] Standard one-letter amino acid abbreviation (see list of Abbreviations and Acronyms).

Table 12 Primer sequences for PCR analysis of Bt corn[a]

Primer	Sequence (5′–3′)[a]	Length of amplicon (bp)
Invertase	CCGCTGTATCACAAGGGCTGGTACC	
Invertase	GGAGCCCGTGTAGAGCATGACGATC	226
Cry1A(b)	ACCATCAACAGCCGCTACAACGACC	
Cry1A(b)	TGGGGAACAGGCTCACGATGTCCAG	184

[a] Standard one-letter amino acid abbreviation (see list of Abbreviations and Acronyms).

These reference genes demonstrate that the DNA isolated was of sufficient quality and quantity for PCR amplification. It is assumed that in the course of food processing, the species-specific reference gene and the transgene are degraded in a similar manner. It is also assumed that effects of the matrix on PCR amplification will be similar. The reduced amplification efficiency of both genes presumably has no effect on the ratio of their amounts, which reflects the ratio of modified and unmodified DNA.

Negative controls demonstrate the absence of laboratory contamination or sample cross-contamination. DNA extracts from nontransgenic plants, clean buffer and mastermix with no template DNA added are common negative controls that are run concurrently with the test samples in the PCR.

Positive controls demonstrate adequate amplification and may be used to quantify the sensitivity of the reaction. One approach is to add known amounts of reference material [e.g., soybean and corn powder containing 0.1% (w/w) genetically altered material] to the standard PCR and to run these concurrently with the test samples. Plant genomic DNA and GMO genomic DNA may also be used as positive controls in the PCR.

3.2.9 Primer design

Primer design is one of the most important aspects of a robust PCR assay. In general, primers should be designed such that they are not able to form secondary structures such as stemloop or hairpin configurations. A primer must not be complementary at the 3′ end, as this will cause primer dimers to form. All primers should have similar melting temperatures and should not contain stretches of individual nucleotides. There are software programs available to assist in primer design, but it is crucial that primers are tested in the assay, especially in a multiplex system.

3.2.10 PCR confirmatory techniques

Presented below are four increasingly stringent confirmatory techniques for PCR and a brief discussion of considerations, limitations and advantages of each. These four techniques are agarose gel electrophoresis, restriction analysis, Southern blotting and sequencing.

Agarose gel electrophoresis can be used to determine whether the PCR amplicon is the expected size. The density of the gel should be chosen to ensure resolution of

the amplicon, and the molecular weight marker should be chosen to encompass the expected size range of the amplicon. A limitation to this approach is that it gives an indication only of the size of the amplification product, not its identity. An advantage is that the technique is quick and easy, allowing for screening of many samples within a short period of time.

Restriction analysis utilizes known restriction enzyme cleavage sites within the DNA sequence of interest. Knowing the sequence of the target PCR product, one can cleave the DNA with appropriate restriction enzymes and separate those fragments by agarose gel electrophoresis. As with agarose gel electrophoresis, the density of the gel and molecular weight markers must be chosen to appropriately resolve and identify the size of the resultant DNA fragments. This type of analysis will give an indirect indication of the identity of the amplicon based solely on common restriction sites and size. Using the known restriction enzyme cleavage sites gives more conclusive data than simple gel electrophoresis, because the recognition site must be present to produce a DNA fragment of the predicted size. Restriction analysis is easily performed on a large number of samples in a short period of time.

Southern blotting consists of agarose gel electrophoresis of the PCR product followed by transfer of the DNA to a solid support matrix, and hybridization with a labeled DNA probe. This technique allows for the determination of the amplicon size and infers specificity related to the DNA probe. As with agarose gel electrophoresis, the density of the gel and molecular weight markers must be chosen appropriately for the size of amplicon being analyzed. It is important that the DNA probe be adequately characterized to ensure its specificity to the targeted DNA sequence. The Southern blotting technique is a lengthy process, but this technique allows for the confirmation of reactivity to a specific DNA probe, giving more confidence about the identity of the PCR product.

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Sequencing the amplicon is the most conclusive confirmatory technique. The main consideration is that the DNA must be appropriately purified to achieve unambiguous sequencing data. However, sequencing requires expensive laboratory equipment that may not be available in all labs. Sequencing does not depend upon the specificity of a probe, or restriction enzyme, but gives a direct identification of the amplicon of interest.

3.3 Basic principles of real-time PCR

Real-time quantitative PCR offers an approach to DNA detection by monitoring the accumulation of PCR products as they are generated. A single copy of a target DNA sequence can yield 2^n copies after n cycles. Hence, theoretically, there is a relationship between starting copy number and amount of PCR product at any given cycle (Figure 20, line A). In reality, replicate reactions often yield widely different amounts of PCR product (Figure 20, line B). This is due to reagents and enzyme activity limiting the reaction. It is difficult to quantify the starting amount of target DNA based on the endpoint. Real-time PCR has the potential to decrease the variability of the measurement by using kinetic rather than endpoint analysis of the PCR process.

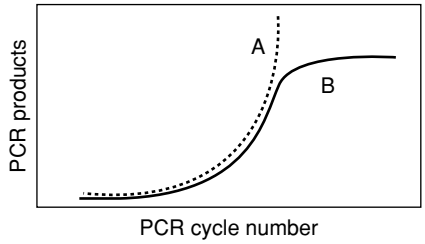


Figure 20 Plot of PCR products produced against the number of amplification cycles. (A) Theoretical PCR product amplified and (B) actual PCR product amplified

3.3.1 Intercalating dyes

The first real-time systems detected PCR products as they were accumulating using DNA binding dyes, such as ethidium bromide.[124,125] UV radiation was applied during thermal cycling, resulting in increasing amounts of fluorescence, which was captured with a charge-coupled device (CCD) camera. The increase in fluorescence ($\Delta_n R$) was plotted against cycle number to give a picture of the kinetics of the PCR process rather than merely assaying the amount of PCR product that had accumulated at a fixed endpoint. These binding dyes are nonspecific, because a fluorescent signal is generated for any double-stranded DNA present. The presence of double-stranded DNA could be due to mispriming or the formation of primer dimer artifacts rather than specific amplification of the target sequence. Nonetheless, DNA binding dyes are very useful in real-time PCR when specificity is not a concern. Examples of commonly used intercalators are ethidium bromide and SYBR Green.[126]

3.3.2 Fluorogenic probes

With fluorogenic probes, it is possible to detect specifically the target sequence in real-time PCR because specific hybridization is required to generate fluorescence. A typical fluorogenic probe is an oligonucleotide with both a reporter and a quencher dye attached. The probe typically binds to the target sequence between the two primers. The proximity of the quencher in relation to the reporter molecule reduces the Forster resonance energy transfer (FRET) of the fluorescent signal emitted from the reporter. There are also a wide range of fluorophores/quenchers and several different hybridization probe strategies available (Table 13).

The three main categories of hybridization probes for real-time PCR are (1) cleavage based assays such as TaqMan, (2) displaceable probe assays such as Molecular Beacons and (3) probes which are incorporated directly into primers such as Scorpions.

Table 13 Common fluorophores/quenchers

DABCYL	4-(4-Dimethylaminophenylazo)benzoic acid
FAM	Fluorescein
TET	Tetrachloro-6-carboxyfluorescein
HEX	Hexachloro-6-carboxyfluorescein
TAMRA	Tetramethylrhodamine
ROX	Rhodamine-X

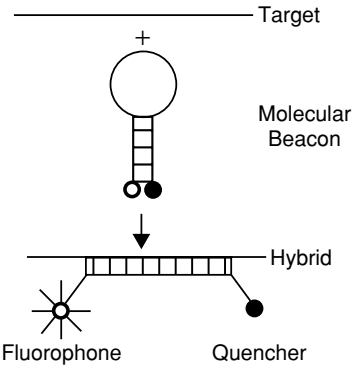


Figure 21 Schematic of the Molecular Beacon

3.3.3 Examples of fluorescent PCR systems

The TaqMan system is also called the fluorogenic 5′ nuclease assay. This technique uses the 5′ nuclease activity of Taq polymerase to cleave an internal oligonucleotide probe. The probe is labeled with both a fluorescent reporter dye and a quencher. The assay results are detected by measuring changes in fluorescence that occur during the amplification cycle as the fluorescent probe is cleaved, uncoupling the dye and quencher labels. The increase in the fluorescent signal is proportional to the amplification of target DNA.

The Molecular Beacons system uses probes that are configured in the shape of a stem and loop. In this conformation, the probe is 'dark' (background level fluorescence) because the stem hybrid keeps the fluorophore in close proximity to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, resulting in increased fluorescence proportional to the amplification of target DNA (Figure 21).

The Scorpions system combines a primer, a specific hybridization probe, fluorophore and quencher in a single molecule. When the Scorpions primer is in a stem and loop conformation, the fluorophore and quencher are in close proximity. The initial heating step denatures the template and also the stem of the Scorpions primer. The primer anneals to the template and strand elongation occurs, producing a PCR amplicon. This double-stranded DNA is denatured and the specific hybridization probe (sequence originally within the loop of the stem/loop) reaches back and hybridizes to the PCR product, binding to the target in an intramolecular manner. The new conformation separates the fluorophore and quencher, resulting in an increase in the fluorescent signal that is proportional to the amplification of target DNA.[127]

3.3.4 Quantitative results/data interpretation

A method for quantitation of the amount of target involves measuring threshold cycle (C_T) and use of a calibration curve to determine starting copy number. The parameter C_T is defined as the fractional cycle number at which the fluorescence passes a fixed threshold. A plot of the log of initial target copy number for a set of standards versus C_T is a straight line (Figure 22).[125] Thus, when the percentage of GMOs in the sample

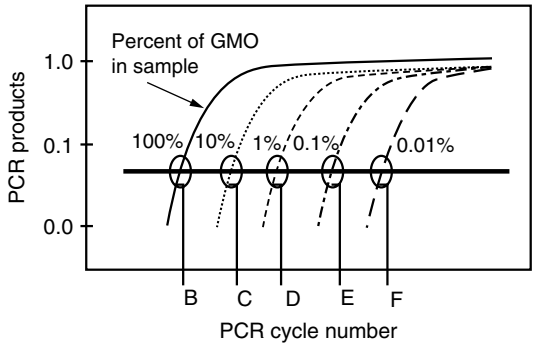


Figure 22 Real-time quantitation of PCR products. The straight line represents the threshold fluorescence value. Each curved line is a plot of the PCR products formed against the number of cycles for different samples. For samples containing 100% GMO, only *B* cycles are required to reach the threshold fluorescence. Samples containing 0.01% GMO will require *F* cycles before the threshold is attained

is 100%, the threshold fluorescence will be reached after only *B* cycles, whereas the sample containing 0.01% of GMO will reach the threshold after *F* cycles.

The use of C_T values also expands the dynamic range of quantitation because data are collected for every PCR cycle. A linear relationship between C_T and initial DNA amount has been demonstrated over five orders of magnitude, compared with the one or two orders of magnitude typically observed with an endpoint assay.[126]

3.4 Applications of PCR to agricultural biotechnology

3.4.1 Research and development

The PCR technique is very useful during all stages of the research and development of biotech crops. PCR analysis is used for gene discovery, event selection, screening, transformant identification, line selection and plant breeding. Quantitative real-time PCR is used to determine the number of transgene copies inserted in experimental plants.

3.4.2 Regulatory submissions

PCR is used to support regulatory submissions. For example, a petition for nonregulated status for a biotech crop must contain the following information:

- rationale for development of product
- description of crop
- description of transformation system
- the donor genes and regulatory sequences
- genetic analysis and agronomic performance
- environmental consequences of introduction
- adverse consequences of introduction
- references.

PCR analysis is one of the techniques used to generate data for the genetic analysis requirement.

3.4.3 Food and commodity testing

There are commercial testing laboratories that offer PCR testing of commodities and food for GMO content. Testing of bulk commodities such as corn grain requires a large sample size. A 2500-g sample is required to have a 99.9% probability of detecting 0.1% GMO content in a sample. The sampling strategy must produce a statistically valid sample for the test results to be meaningful. The entire 2500-g sample would typically be ground, and duplicate 10-g subsamples of raw corn or soy would be extracted. For processed or mixed foods, duplicate 2-g subsamples would typically be extracted.

These PCR laboratories often offer GMO screening, specific tests for certain commercial GMOs and real-time quantitative testing. The different approaches vary widely in cost and the choice would depend on the testing objective.

3.5 Recent advances in nucleic acid amplification and detection

Many nucleic acid detection strategies use target amplification, signal amplification or both. Invader, branched DNA (bDNA) and rolling circle amplification (RCA) are three approaches.

Invader is a signal amplification approach. This cleavage-based assay uses two partially overlapping probes that are cleaved by an endonuclease upon binding of the target DNA. The Invader system uses a thermostable endonuclease and elevated temperature to evoke about 3000 cleavage events per target molecule. A more sensitive homogeneous Invader assay exists in which the cleaved product binds to a second probe containing a fluorophore and quencher. The second probe is also cleaved by endonuclease, generating 10^7 fluorescence events for each target molecule, which is sensitive enough to detect less than 1000 targets.[128]

bDNA achieves signal amplification by attaching many signal molecules (such as alkaline phosphatase) to a DNA dendrimer. Several tree-like structures are built in each molecular recognition event. The Quantiplex bDNA assay (Chiron) uses a dioxetane substrate for alkaline phosphatase to produce chemiluminescence.[127]

The linear RCA method can use both target and signal amplification. A DNA circle (such as a plasmid, circular virus or circular chromosome) is amplified by polymerase extension of a complementary primer. Up to 10^5 tandemly repeated, concantemerized copies of the DNA circle are generated by each primer, resulting in one single-stranded, concantemerized product.[129]

4 Biosensors: immunosensors

The development of immunosensors is one of the most active research areas in immunodiagnostics. A large number of immunosensors, which combine the sensitivity and specificity of immunoassays with physical signal transduction, have been developed

in recent years for pesticide analysis. A classical biosensor consists of three components, including a receptor (an antibody or binding protein), a transducer (e.g., an optical fiber or electrode) and signal processing electronics. The receptor is usually immobilized to the transducer surface, which enables it to detect interaction with analyte molecules. In contrast to immunoassays, immunosensors commonly rely on the reuse of the same receptor surface for many measurements. Direct signal generation potentially enables real-time monitoring of analytes, thus making immunosensors suitable tools for continuous environmental monitoring.

There are several classes and subclasses of immunosensors, each with advantages for environmental analysis. Piezoelectric sensors (including bulk acoustic and surface acoustic wave) use an external alternating electric field to directly measure the antibody–antigen interaction. Electrochemical sensors (including potentiometric, amperometric, capacitative and conductimetric) may offer inexpensive analytical alternatives for effluent monitoring.[130,131] Optical sensors (including fiber-optic, evanescent wave biosensors and Mach–Zehnder interferometer sensors) measure the absorption or emission of a wavelength of light and base detection on fluorescence, absorbance, luminescence or total internal reflectance fluorescence.[132,133]

Surface plasmon resonance (SPR) is an optical electronic technique in which an evanescent electromagnetic field generated at the surface of a metal conductor is excited by light of a certain wavelength at a certain angle. An immunosensor has been developed for the detection of atrazine using SPR.[134] Moreover, a grating coupler immunosensor was evaluated for the measurement of four *s*-triazine herbicides.[135] One could detect terbutryn in the range 15–60 nM using this biosensor. Because antibody-based biosensors have no associated catalytic event to aid in transduction, they are far more complex than enzyme-based biosensors. In addition, they do not release their ligand quickly, leading to a slow response. Theoretically, biosensors are capable of continuous and reversible detection, but reversibility is difficult to achieve in practice because sensitive antibody–antigen interactions have high affinity constants. Because cost and time are critical factors in environmental monitoring, it is likely that the development of small-probe antibody-based biosensors yielding continuous readouts of an analyte at low concentration will not be rapid. However, research in the sensor field is certain to give improvements in many aspects of immunoassay technology, and antibody–hapten and receptor–ligand binding assays are being coupled to biological and physical transducers in many ingenious ways.

4.1 Biological transducers

With enzymes, binding proteins or receptors, it is attractive to use biological transduction. A simple example is acetylcholinesterase for the detection of organophosphate and carbamate insecticides. Binding of these materials to the enzyme inhibits it, thus blocking substrate turnover. Similar approaches can be used for herbicide detection. Coupling a receptor to its natural responsive element also can provide a valuable biosensor. This could be induction of natural proteins such as vitellogenen by estradiol or the responsive element could be moved upstream of luciferase, a fluorescent protein or other easily detected biological molecules.[136]

5 Conclusion

As described by Hammock and Mumma,[8] there are many unique applications for immunodiagnostics in pesticide chemistry. Such uses include human monitoring, field monitoring, analysis of chirality, analysis of complex molecules and analytical problems where large numbers of samples must be processed quickly. Such applications are expanding as we see the development of more complex and nonvolatile pesticide chemicals and the need to monitor polar metabolites, environmental degradation products and GMOs. However, other analytical technologies are improving. For example, liquid chromatography/mass spectrometry (LC/MS) technologies increasingly can handle complex molecules and, like immunoassay, tandem mass spectrometry (MS/MS) technologies avoid the need for many cleanup steps. Hence, many of the traditional applications of immunoassay will be replaced by other technologies if immunochemistry remains static. Active research on new formats and new applications of immunoassays argues for a continued place for the technology in the repertoire of environmental chemists. Coupled immunochemical techniques are promising where, for example, antibodies are used as sensitive, selective detection systems for HPLC[137] or for immunoaffinity procedures preceding MS[138] or other analyses.

Although immunoassays can compete effectively with other technologies in the analysis of small molecules, a major strength of the technology is in the analysis of peptides and proteins. With the expanded use of GMOs in agriculture, all of which to date are expressing novel proteins, there is a new and important application for immunoassay. The technology will be important for GMO development, product stewardship and quality control. With some public concern over the safety of GMOs, there is a commercial need for high-throughput and for field analysis of food products for GMO content. High throughput and field analysis are two major strengths of immunoassay technology, making it an ideal technology for monitoring indicators of food quality. Food quality monitoring, then, represents a major market for this technology.

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6 Abbreviations

A	adenine
Ab	antibody
ACCD	1-aminocyclopropane-1-carboxylic acid deaminase
ACCS	aminocyclopropane carboxylic acid synthase
Ag	antigen
ALS	acetolactate synthase
bDNA	branched DNA
bp	base pairs
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
C	cytosine
CaMV	cauliflower mosaic virus
CCD	charge-coupled device
CD	compact disk

CMC	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (same as Morpho CDI)
CMV	cucumber mosaic virus
C_T	threshold cycle
DAM	DNA adenine methylase
DCC	dicyclohexylcarbodiimide
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FDA	Food and Drug Administration
FIIA	flow injection immunoassay
FRET	Forster resonance energy transfer
G	guanine
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
GE	genetically engineered
GLC	gas–liquid chromatography
GM	genetically modified
GMO	genetically modified organism
GOX	glyphosate oxidoreductase
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
I_{50}	the concentration of analyte that inhibits the immunoassay by 50%
IgG	immunoglobulin G
K_A	equilibrium binding constant for the binding of analyte to antibody
K_H	equilibrium binding constant for the binding of hapten to antibody
KLH	keyhole limpet hemocyanin
LC/MS	liquid chromatography/mass spectrometry
LLD	lower limit of detection
LOQ	limit of quantitation
LPH	horseshoe crab hemocyanin
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MBS	*m*-maleimidobenzoyl-*N*-hydroxysuccinimide
Morpho CDI	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (same as CDI)
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NHS	*N*-hydroxysuccinimide
NPTII	neomycin phosphotransferase II

OD	optical density
PAT	phosphinothricin acetyltransferase
PBA	phenoxybenzoic acid
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PG	polygalacturonase
PRSV	papaya ringspot virus
QC	quality control
RCA	rolling circle amplification
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance
T	thymine
T_a	annealing temperature
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
T_m	melting temperature
Ti	tumor-inducing
TOF	time-of-flight
TPS	template preparation solution
USDA	United States Department of Agriculture
USDA GIPSA	United States Department of Agriculture Grain Inspection Protection Service
USEPA	United States Environmental Protection Agency
UV	ultraviolet
UV/VIS	ultraviolet/visible
WMV2	watermelon mosaic virus2
ZYMV	zucchini yellow mosaic virus
λ_{max}	wavelength of maximum absorption

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References

1. L. Landsteiner, 'The Specificity of Serological Reactions,' Harvard University Press, Cambridge, MA (1945).
2. R.S. Yalow and S.A. Berson, *Nature (London),* **184**, 1648 (1959).
3. R.S. Yalow and S.A. Berson, *J. Clin. Invest.,* **39**, 1157 (1960).
4. E.R. Centeno and W.J. Johnson, *Int. Arch. Allergy Appl. Immunol.,* **37**, 1 (1970).
5. J.J. Langone and H. Van Vunakis, *Res. Commun. Chem. Pathol. Pharmacol.,* **10**, 163 (1975).

6. C D. Ercegovich, R.P. Vallejo, R.R. Gettig, L. Woods, E.R. Bogus, and R.O. Mumma, *J. Agric. Food Chem.,* **29**, 559 (1981).
7. E. Engvall and P. Perlmann, *J. Immunol.,* **109**, 129 (1972).
8. B.D. Hammock and R.O. Mumma, 'Potential of immunochemical technology for pesticide analysis,' in "Pesticide Analytical Methodology," ed. J.J. Harvey and G. Zweig, American Chemical Society, Washington, DC, pp. 321–352 (1980).
9. S.J. Gee, A.D. Lucas, and B.D. Hammock, 'Using immunochemical methods to analyze for biomarkers of exposure,' in "Methods of Pesticide Exposure Assessment," ed. P.B. Curry, S. Iyengar, P. Maloney, and M. Marconi, Plenum Press, New York, pp. 143–155 (1995).
10. A.S. Harris, I. Wengatz, M. Wortberg, S.B. Kreissig, S.J. Gee, and B.D. Hammock, 'Development and application of immunoassays for biological and environmental monitoring,' in "Multiple Impacts on Ecosystems," ed. J.J. Cech, B.W. Wilson, and D.G. Crosby, Lewis, Boca Raton, FL, pp. 135–153 (1998).
11. M. Oellerich, *J. Clin. Chem. Clin. Biochem.,* **18**, 197 (1980).
12. M.-C. Hennion and D. Barcelo, *Anal. Chim. Acta,* **362**, 3 (1998).
13. J.K. Lee, K.C. Ahn, O.S. Park, S.Y. Kang, and B.D. Hammock, *J. Agric. Food Chem.,* **49**, 2159 (2001).
14. P. Tijssen, 'Practice and Theory of Enzyme Immunoassays,' Elsevier, Amsterdam (1985).
15. A. Voller, D.E. Bidwell, and A. Bartlett, *Bull. World Health Org.,* **53**, 55 (1976).
16. J.A. Gabaldon, A. Maquieira, and R. Puchades, *Crit. Rev. Food Sci. Nutr.,* **39**, 519 (1999).
17. W.A. Matthews and M. Haverly, *Food Agric. Immunol.,* **7**, 153 (1995).
18. E.P. Meulenberg, L.G. de Vree, and J. Dogterom, *Anal. Chim. Acta,* **399**, 143 (1999).
19. C. Mouvet, S. Broussard, R. Jeannot, C. Maciag, R. Abuknesha, and G. Ismail, *Anal. Chim. Acta,* **311**, 331 (1995).
20. D. Rodbard, 'Mathematics and statistics of ligand assays: an illustrated guide,' in "Ligand Assay: Analysis of International Developments on Isotopic and Nonisotopic Immunoassay," ed. J. Langan and J.J. Clapp, Masson, New York, pp. 45–99 (1981).
21. H.E. Grotjan and B.A. Keel, 'Data Interpretation and Quality Control,' in "Immunoassay," ed. E.P. Diamandis and T.K. Christopoulos, Academic Press, San Diego, pp. 51–95 (1996).
22. D. Rodbard, *Anal. Biochem.,* **90**, 1 (1978).
23. United States Department of Agriculture, 'Grain Inspection Handbook, Book 1, Grain Sampling,' USDA, Washington, DC (1995).
24. J.W. Stave, *Food Control,* **10**, 367 (1999).
25. G.J. Rogan, Y.A. Dudin, T.C. Lee, K.M. Magin, J.D. Astwood, N.S. Bhakta, J.N. Leach, P.R. Stanley, and R.L. Fuchs, *Food Control,* **10**, 407 (1999).
26. Envirologix, Portland, ME, **http://www.envirologix.com**.
27. F. Jung, S.J. Gee, R.O. Harrison, M.H. Goodrow, A.E. Karu, A.L. Braun, Q.X. Li, and B.D. Hammock, *Pestic. Sci.,* **26**, 303 (1989).
28. M.T. Muldoon and J.O. Nelson, *J. Agric. Food Chem.,* **42**, 1686 (1994).
29. J.P. Sherry, *Crit. Rev. Anal. Chem.,* **23**, 217 (1992).
30. M.P. Marco, S.J. Gee, H.M. Cheng, Z.Y. Liang, and B.D. Hammock, *J. Agric. Food Chem.,* **41**, 423 (1993).
31. D.P. McAdam, A.S. Hill, H.L. Beasley, and J.H. Skerritt, *J. Agric. Food Chem.,* **40**, 1466 (1992).
32. P. Schneider and B.D. Hammock, *J. Agric. Food Chem.,* **40**, 525 (1992).
33. G.P. Holland and M.W. Steward, *J. Immunol. Methods,* **138**, 245 (1991).
34. M.J. Khosravi and A. Papanastasiou-Diamandi, *Clin. Chem.,* **39**, 256 (1993).
35. G. Shan, W.R. Leeman, D.W. Stoutamire, S.J. Gee, D.P.Y. Chang, and B.D. Hammock, *J. Agric. Food Chem.,* **48**, 4032 (2000).
36. G. Shan, D.W. Stoutamire, I. Wengatz, S.J. Gee, and B.D. Hammock, *J. Agric. Food Chem.,* **47**, 2145 (1999).
37. N. Lee, H.L. Beasley, and J.H. Skerritt, *J. Agric. Food Chem.,* **46**, 535 (1998).
38. N. Lee, D.P. McAdam, and J.H. Skerritt, *J. Agric. Food Chem.,* **46**, 520 (1998).
39. T. Watanabe, G. Shan, D.W. Stoutamire, S.J. Gee, and B.D. Hammock, *Anal. Chim. Acta,* **444**, 119 (2001).
40. W.H. Newsome, J.M. Yeung, and P.G. Collins, *J. AOAC Int.,* **70**, 381 (1993).
41. M.H. Goodrow, R.O. Harrison, and B.D. Hammock, *J. Agric. Food Chem.,* **38**, 990 (1990).

42. A.S. Hill, D.P. McAdam, S.L. Edward, and J.H. Skerritt, *J. Agric. Food Chem.,* **70**, 381 (1993).
43. J.R. Sanborn, S.J. Gee, S.D. Gilman, Y. Sugawara, A.D. Jones, J. Rogers, F. Szurdoki, L.H. Stanker, D.W. Stoutamire, and B.D. Hammock, *J. Agric. Food Chem.,* **46**, 2407 (1998).
44. Y. Sugawara, S.J. Gee, J.R. Sanborn, S.D. Gilman, and B.D. Hammock, *Anal. Chem.,* **70**, 1092 (1998).
45. Y.-W. Chiu, R.E. Carlson, K.L. Marcus, and A.E. Karu, *Anal. Chem.,* **67**, 3829 (1995).
46. P. Schneider, M.H. Goodrow, S.J. Gee, and B.D. Hammock, *J. Agric. Food Chem.,* **42**, 413 (1994).
47. S.I. Wie and B.D. Hammock, *J. Agric. Food Chem.,* **32**, 1294 (1984).
48. R.O. Harrison, M.H. Goodrow, and B.D. Hammock, *J. Agric. Food Chem.,* **39**, 122 (1991).
49. A.E. Karu, M.H. Goodrow, D.J. Schmidt, B.D. Hammock, and M.W. Bigelow, *J. Agric. Food Chem.,* **42**, 301 (1994).
50. G. Shan, W.R. Leeman, S.J. Gee, J.R. Sanborn, A.D. Jones, D.P.Y. Chang, and B.D. Hammock, *Anal. Chim. Acta,* **444**, 169 (2001).
51. G.T. Hermanson, 'Bioconjugate Techniques,' Academic Press, San Diego (1996).
52. B.F. Erlanger, *Methods Enzymol.,* **70**, 85 (1980).
53. M. Brinkley, *Bioconj. Chem.,* **3**, 2 (1992).
54. I. Wengatz, D.W. Stoutamire, S.J. Gee, and B.D. Hammock, *J. Agric. Food Chem.,* **46**, 2211 (1998).
55. B.F. Erlanger, F. Borek, S.M. Beiser, and S. Lieberman, *J. Biol. Chem.,* **228**, 713 (1957).
56. B.F. Erlanger, F. Borek, S.M. Beiser, and S. Lieberman, *J. Biol. Chem.,* **234**, 1090 (1959).
57. N. Zegers, K. Gerritse, C. Deen, W. Boersma, and E. Claassen, *J. Immunol. Methods,* **130**, 195 (1990).
58. A.F.S.A. Habeeb, *Anal. Biochem.,* **14**, 328 (1966).
59. A.D. Lucas, H.K.M. Bekheit, M. Goodrow, A.D. Jones, S. Kullman, F. Matsumura, J.E. Woodrow, J.N. Seiber, and B.D. Hammock, *J. Agric. Food Chem.,* **41**, 1523 (1993).
60. I. Wengatz, R.D. Schmid, S. Kreissig, C. Wittmann, B. Hock, A. Ingendoh, and F. Hellenkamp, *Anal. Lett.,* **25**, 1983 (1992).
61. J.M. Peeters, T.G. Hazendonk, E.C. Beuvery, and G.I. Tesser, *J. Immunol. Methods,* **120**, 133 (1989).
62. L.G. Bachas and M.E. Meyerhoff, *Anal. Biochem.,* **156**, 223 (1986).
63. A.M. Campbell, 'Monoclonal Antibody and Immunosensor Technology: the Production and Application of Rodent and Human Monoclonal Antibodies,' Elsevier, Amsterdam (1991).
64. J.W. Goding, 'Monoclonal Antibodies: Principles and Practice: Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology,' Academic Press, London (1996).
65. G. Kohler and C. Milstein, *Nature (London),* **256**, 495 (1975).
66. E. Harlow and D. Lane, 'Antibodies: a Laboratory Manual,' Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988).
67. C.A. Williams and M.W. Chase, 'Methods in Immunology and Immunochemistry,' Academic Press, New York (1967).
68. G. Winter and C. Milstein, *Nature (London),* **349**, 293 (1991).
69. A. Pluckthun and A. Skerra, *Methods Enzymol.,* **178**, 497 (1989).
70. J. Maynard and G. Georgiou, *Annu. Rev. Biomed. Eng.,* **2**, 339 (2000).
71. S. Lange, J. Schmitt, and R.D. Schmid, *J. Immunol. Methods,* **255**, 103 (2001).
72. N.L. Tout, K.Y. Yau, J.T. Trevors, H. Lee, and J.C. Hall, *J. Agric. Food Chem.,* **49**, 3628 (2001).
73. J. Quinn, P. Patel, B. Fitzpatrick, B. Manning, P. Dillon, S. Daly, R. Okennedy, M.J. Alcocer, H. Lee, M.R. Morgan, and K. Lang, *Biosens. Bioelectron.,* **14**, 587 (1999).
74. M.J. Alcocer, C. Koyen, H.A. Lee, and M.R. Morgan, *J. Agric. Food Chem.,* **48**, 4053 (2000).
75. C.W. Bell, K.B. Scholthof, G. Zhang, and A.E. Karu, *Gene,* **165**, 323 (1995).
76. S.J. Gee, B.D. Hammock, and J.M. Van Emon, 'Environmental Immunochemical Analysis for Detection of Pesticides and other Chemicals: a User's Guide,' Nyes Publications, Westwood, NJ (1997).
77. D.B. Baker, R.J. Bushway, S.A. Adams, and C. Macomber, *Environ. Sci. Technol.,* **27**, 52 (1993).

DropBooks

78. A.E. Karu, R.O. Harrison, D.J. Schmidt, C.E. Clarkson, J. Grassman, M.H. Goodrow, A. Lucas, B.D. Hammock, J.M. Van Emon, and R.J. White, 'Monoclonal immunoassay of triazine herbicides: development and implementation,' in "Immunoassays for Trace Chemical Analysis: Monitoring Toxic Chemicals in Humans, Food, and the Environment.," ed. M. Vanderlaan, L.H. Stanker, B.E. Watkins, and D.W. Roberts, American Chemical Society, Washington, DC, pp. 59–77 (1991).
79. J.H. Skerritt and B.E.A. Rani, 'Detection and removal of sample matrix effects in agrochemical immunoassays,' in "Immunoassays for Residue Analysis," ed. R.C. Beier and L.H. Stanker, American Chemical Society, Washington, DC, pp. 29–43 (1996).
80. C.R. Lipton, J.X. Dautlick, G.D. Grothaus, P.L. Hunst, C.A. Magin, F. Mihaliak, M. Rubio, and J.W. Stave, *Food Agric. Immunol.,* **12**, 153 (2000).
81. G.T. Wernimont and W. Spendley, 'Use of Statistics to Develop and Evaluate Analytical Methods,' Association of Official Analytical Chemists, Arlington, VA (1985).
82. R.O. Harrison, A.L. Braun, S.J. Gee, D.J. O'Brian, and B.D. Hammock, *Food Agric. Immunol.,* **1**, 37 (1989).
83. G. Jones, M. Wortberg, S.B. Kreissig, B.D. Hammock, and D.M. Rocke, *Anal. Chim. Acta.,* **313**, 197 (1995).
84. P. Schneider, S.J. Gee, S.B. Kreissig, A.S. Harris, P. Krämer, M.-P. Marco, A.D. Lucas, and B.D. Hammock, 'Troubleshooting during the development and use of immunoassays for environmental monitoring,' in "New Frontiers in Agrochemical Immunoassay," ed. D.A. Kurtz, J.H. Skerritt, and L.H. Stanker, AOAC International, Washington, DC, pp. 103–122 (1995).
85. T.D. Spittler, S.K. Brightman, M.C. Humiston, and D.R. Forney, 'Watershed monitoring in sustainable agriculture studies,' in "Agrochemical Fate and Movement: Perspective and Scale of Study," ed. T.R. Steinheimer, L.J. Ross, and T.D. Spittler, American Chemical Society, Washington, DC, pp. 126–134 (2000).
86. A.E. Walker, R.E. Holman, and R.B. Leidy, *J. Am. Water Resour. Assoc.,* **36**, 67 (2000).
87. T.R. Dombrowski, E.M. Thurman, and G.B. Mohrman, 'A first application of enzyme-linked immunosorbent assay for screening cyclodiene insecticides in ground water,' in "Environmental Immunochemical Methods," ed. J.M. Van Emon, C.L. Gerlach, and J.C. Johnson, American Chemical Society, Washington, DC, pp. 148–154 (1996).
88. H.A. Moye, *Chem. Anal. (NY),* **151**, 211 (1999).
89. B.S. Rutherford, R.C. Gardner, S.D. West, C.K. Robb, and S.C. Dolder, *J. Agric. Food Chem.,* **48**, 4428 (2000).
90. L. Guzzella, A.A. De Paolis, C. Bartone, F. Pozzoni, and G. Giuliano, *Int. J. Environ. Anal. Chem.,* **65**, 261 (1996).
91. S.J. Gee, B.D. Hammock, and J.H. Skerritt, 'Diagnostics for plant agrochemicals–a meeting of chemistry and immunoassay,' in "New Diagnostics in Crop Sciences," ed. J.H. Skerritt and R. Appels, CAB International, Wallingford, pp. 243–276 (1995).
92. R. Galve, M. Nichkova, F. Camps, F. Sanchez-Baeza, and M. P. Marco, *Anal. Chem.,* **74**, 468 (2002).
93. J.M. Van Emon and C.L. Gerlach, *J. Microbiol. Methods,* **32**, 121 (1998).
94. B.S. Ferguson and H.N. Nigg, *BCPC Symp. Proc.,* **65**, 179 (1996).
95. R.E. Biagini, W. Tolos, W.T. Sanderson, G.M. Henningsen, and B. MacKenzie, *Bull. Environ. Contam. Toxicol.,* **54**, 245 (1995).
96. W.T. Sanderson, R.E. Biagini, W. Tolos, G.M. Henningsen, and B. MacKenzie, *Am. Ind. Hyg. Assoc. J.,* **56**, 883 (1995).
97. L.L. Jaeger, A.D. Jones, and B.D. Hammock, *Chem. Res. Toxicol.,* **11**, 342 (1998).
98. J.F. Brady, J. Turner, M.W. Cheung, J.D. Vargo, J.G. Kelly, D.W. King, and A.C. Alemanni, 'An immunochemical approach to estimating worker exposure to atrazine,' in "Triazine Herbicides: Risk Assessment," ed. L.G. Ballantine, J.E. McFarland, and D.S. Hackett, American Chemical Society, Washington, DC, pp. 131–140 (1998).
99. C.A.F. Striley, R.E. Biagini, J.P. Mastin, B.A. MacKenzie, and S.K. Robertson, *Anal. Chim. Acta,* **399**, 109 (1999).
100. G. Shan, I. Wengatz, D.W. Stoutamire, S.J. Gee, and B.D. Hammock, *Chem. Res. Toxicol.,* **12**, 1033 (1999).

101. B.A. Buchholz, E. Fultz, K.W. Haack, J.S. Vogel, S.D. Gilman, S.J. Gee, B.D. Hammock, X. Hui, R.C. Wester, and H.I. Maibach, *Anal. Chem.,* **71**, 3519 (1999).
102. J. Fagan, B. Schoel, A. Haegert, J. Moore, and J. Beeby, *Int. J. Food Sci. Technol.,* **36**, 357 (2001).
103. J. Rudzicka and A. Ivaska, *Anal. Chem.,* **69**, 5024 (1997).
104. P.M. Krämer, A. Franke, and C. Standfuss-Gabisch, *Anal. Chim. Acta,* **399**, 89 (1999).
105. R. Puchades, A. Maquieira, J. Atienza, and A. Montoya, *Crit. Rev. Anal. Chem.,* **23**, 301 (1992).
106. J. Strahan, 'Development and application of an enzyme-linked immunosorbent assay method for the determination of multiple sulfonylurea herbicides on the same microwell plate,' in "Environmental Immunochemical Methods," ed. J.M. Van Emon, C.L. Gerlach, and J.C. Johnson, American Chemical Society, Washington, DC, pp. 65–73 (1996).
107. H. Kido, A. Maquieira, and B.D. Hammock, *Anal. Chim. Acta,* **411**, 1 (2000).
108. C.A. Rowe, S.B. Scruggs, M.J. Feldstein, J.P. Golden, and F.S. Ligler, *Anal. Chem.,* **71**, 433 (1999).
109. K.E. Sapsford, P.T. Charles, C.H.J. Patterson, and F.S. Ligler, *Anal. Chem.,* **74**, 1061 (2002).
110. C. Lohse, L.L. Jaeger, N. Staimer, J.R. Sanborn, A.D. Jones, J. Lango, S.J. Gee, and B.D. Hammock, *J. Agri. Food Chem.,* **48**, 5913 (2000).
111. N. Staimer, S.J. Gee, and B.D. Hammock, *Anal. Chim. Acta,* **444**, 27 (2001).
112. J.C. Johnson, J.M. Van Emon, D.R. Pullman, and K.R. Keeper, *J. Agric. Food Chem.,* **46**, 3116 (1998).
113. S. Wang, R.D. Allan, J.H. Skerritt, and I.R. Kennedy, *J. Agric. Food Chem.,* **46**, 3330 (1998).
114. G. Jones, M. Wortberg, S.B. Kreissig, D.S. Bunch, S.J. Gee, B.D. Hammock, and D.M. Rocke, *J. Immunol. Methods,* **177**, 1 (1994).
115. M. Wortberg, G. Jones, S.B. Kreissig, D.M. Rocke, S.J. Gee, and B.D. Hammock, *Anal. Chim. Acta,* **319**, 291 (1996).
116. M. Wortberg, S.B. Kreissig, G. Jones, D.M. Rocke, and B.D. Hammock, *Anal. Chim. Acta,* **304**, 339 (1995).
117. United States Department of Agriculture, Washington, DC, **http://www.usda.gov/agencies/biotech/research.html.**
118. S.R. Barnum, 'Biotechnology,' Wadsworth, Belmont, CA (1998).
119. GeneScan Europe, Freiburg, **http://www.genescan.com.**
120. United States Food and Drug Administration, Washington, DC, **http://www.cfsan.fda.gov/~lrd/biocon.html.**
121. C. Tengel, P. Schussler, E. Setzke, J. Balles, and M. Sprenger-Haussels, *Biotechniques,* **31**, 426 (2001).
122. D. Thomson and R. Henry, *Biotechniques,* **19**, 394 (1995).
123. Eppendorf Netheler Hinz, Cambridge, **http://eppendorf.com.**
124. R. Higuchi, G. Dollinger, P.S. Walsh, and R. Griffith, *Biotechnology,* **10**, 413 (1992).
125. R. Higuchi, C. Fockler, G. Dollinger, and R. Watson, *Biotechnology,* **11**, 1026 (1993).
126. Applied Biosystems, Foster City, CA, **http://www.appliedbiosystems.com.**
127. B. Schweitzer and S. Kingsmore, *Curr. Opin. Biotechnol.,* **12**, 21 (2001).
128. J. Hall, P. Eis, S. Law, L. Reynaldo, J. Prudent, D. Marshall, H. Allawi, A. Mast, J. Dahlberg, R. Kwiatkowski, M. de Arruda, B.P. Neri, and V.I. Lyamichev, *Proc. Natl. Acad. Sci. USA,* **97**, 8272 (2000).
129. P. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. Thomas, and D. Ward, *Nature Genet.,* **19**, 225 (1998).
130. R.W. Keay and C.J. MacNeil, *Biosens. Bioelectron.,* **13**, 963 (1998).
131. A.J. Baumner and R.D. Schmid, *Bull. Environ. Contam. Toxicol.,* **54**, 245 (1998).
132. M.A. Gonzalez-Martinez, R. Puchades, A. Maquieira, I. Ferrer, M.P. Marco, and D. Barcelo, *Anal. Chim. Acta,* **386**, 201 (1999).
133. E.F. Schipper, A.J.H. Bergevoet, R.P.H. Kooyman, and J. Greve, *Anal. Chim. Acta,* **341**, 171 (1997).
134. M. Minunni and M. Mascini, *Anal. Lett.,* **26**, 1441 (1993).
135. F.F. Bier and R.D. Schmid, *Biosens. Bioelectron.,* **9**, 125 (1994).
136. M.S. Denison, W.J. Rogers, M. Fair, M. Ziccardi, G. Clark, A.J. Murk, and A. Brouwer, *Organohal. Compd.,* **27**, 280 (1996).
137. P.M. Kramer, Q.X. Li, and B.D. Hammock, *J. AOAC Int.,* **77**, 1275 (1994).

DropBooks

138. J.K. Huwe, W.L. Shelver, L.H. Stanker, D.G.J. Patterson, and W.E. Turner, *J. Chromatogr. B,* **15**, 285 (2001).
139. S. Sadakane, T. Sakai, A. Hayashi, and H. Ohkawa, *J. Pestic. Sci.,* **23**, 410 (1998).
140. S. Ben-Rejeb, N. Fischer-Durand, A. Martel, F. Le Goffic, J.F. Lawrence, J.M. Yeung, and M.A. Abbott, *Int. J. Environ. Anal. Chem.,* **69**, 13 (1998).
141. E. Simon, D. Knopp, P.B. Carrasco, and R. Niessner, *Food Agric. Immunol.,* **10**, 105 (1998).
142. E. Welzig, H. Pichler, R. Krska, D. Knopp, and R. Niessner, *Int. J. Environ. Anal. Chem.,* **78**, 279 (2000).
143. J.K. Lee, K.C. Ahn, O.S. Park, Y.K. Ko, and D.-W. Kim, *J. Agric. Food Chem.,* **50**, 1791 (2002).
144. E.V. Yazynina, A.V. Zherdev, S.A. Eremin, V.A. Popova, and B.B. Dzantiev, *Appl. Biochem. Microbiol.,* **38**, 9 (2002).
145. M.W. Shankle, D.R. Shaw, and M. Boyette, *Weed Technol.,* **15**, 669 (2001).
146. G. Hegedus, I. Belai, and A. Szekacs, *Anal. Chim. Acta,* **421**, 121 (2000).
147. U.I. Garimella, G.K. Stearman, and M.J. Wells, *J. Agric. Food Chem.,* **48**, 5874 (2000).
148. S.R. Webb and J.C. Hall, *J. Agric. Food Chem.,* **48**, 1210 (2000).
149. S.R. Webb and J.C. Hall, *J. AOAC Int.,* **84**, 143 (2001).
150. M. Winklmair, M.G. Weller, J. Mangler, B. Schlosshauer, and R. Niessner, *Fresenius' J. Anal. Chem.,* **358**, 614 (1997).
151. J. Leszczynska, J. Maslowska, and A. Owczarek, *Pol. J. Food Nutr. Sci.,* **7**, 733 (1998).
152. L. Bruun, C. Koch, B. Pedersen, M.H. Jakobsen, and J. Aamand, *J. Immunol. Methods,* **240**, 133 (2000).
153. A.I. Krasnova, S.A. Eremin, M. Natangelo, S. Tavazzi, and E. Benfenati, *Anal. Lett.,* **34**, 2285 (2001).
154. A. Navas-Diaz, F. Garcia-Sanchez, J. Lovillo, and J.A. Gonzalez-Garcia, *Anal. Chim. Acta,* **321**, 219 (1996).
155. R.J. Bushway and B.S. Ferguson, *BCPC Symp. Proc.,* **65**, 317 (1996).
156. B.D. Johnson and J.C. Hall, *J. Agric. Food Chem.,* **44**, 488 (1996).
157. F. Szurdoki, A. Szekacs, H.M. Le, and B.D. Hammock, *J. Agric. Food Chem.,* **50**, 29 (2002).
158. G. Giraudi, C. Giovannoli, C. Baggiani, I. Rosso, P. Coletto, A. Vanni, M. Dolci, and G. Grassi, *Anal. Commun.,* **35**, 183 (1998).
159. S. Wang, R.D. Allan, J.H. Skerritt, and I.R. Kennedy, *J. Agric. Food Chem.,* **47**, 3416 (1999).
160. H.L. Beasley, A. Pash, S.L. Guihot, and J.H. Skerritt, *Food Agric. Immunol.,* **12**, 203 (2000).
161. J.V. Mercader and A. Montoya, *J. Agric. Food Chem.,* **47**, 1285 (1999).
162. A. Abad, M.J. Moreno, and A. Montoya, *J. Agric. Food Chem.,* **47**, 2475 (1999).
163. A. Abad, M.J. Moreno, R. Pelegri, M.I. Martinez, A. Saez, M. Gamon, and A. Montoya, *J. Chromatogr. A,* **833**, 3 (1999).
164. M.J. Moreno, A. Abad, R. Pelegri, M.J. Martinez, A. Saez, M. Gamon, and A. Montoya, *J. Agric. Food Chem.,* **49**, 1713 (2001).
165. J.J. Manclus, J. Primo, and A. Montoya, *J. Agric. Food Chem.,* **42**, 1257 (1994).
166. J.J. Manclus, J. Primo, and A. Montoya, *J. Agric. Food Chem.,* **44**, 4052 (1996).
167. P. Wyss, M. Bolsinger, and C. Pfister, *Anal. Lett.,* **29**, 2363 (1996).
168. W.T. Jones, D. Harvey, S.D. Jones, G.B. Ryan, H. Wynberg, W. Ten Hoeve, and P.H.S. Reynolds, *Food Agric. Immunol.,* **7**, 9 (1995).
169. S. Pullen and B. Hock, *Anal. Lett.,* **28**, 781 (1995).
170. S. Pullen and B. Hock, *Anal. Lett.,* **28**, 765 (1995).
171. M. Nakata, A. Fukushima, and H. Ohkawa, *Pestic. Manage. Sci.,* **57**, 269 (2001).
172. S. Oepkemeier, S. Schreiber, D. Breuer, G. Key, and W. Kleibohmer, *Anal. Chim. Acta,* **393**, 103 (1999).
173. W. Ten Hoeve, H. Wynberg, W.T. Jones, D. Harvey, G.B. Ryan, and P.H.S. Reynolds, *Bioconj. Chem.,* **8**, 257 (1997).
174. M.J.C. Alcocer, P.P. Dillon, B.M. Manning, C. Doyen, H.A. Lee, S.J. Daly, R. O'Kennedy, and M.R.A. Morgan, *J. Agric. Food Chem.,* **48**, 2228 (2000).
175. E. Watanabe, Y. Kanzaki, H. Tokumoto, R. Hoshino, H. Kubo, and H. Nakazawa, *J. Agric. Food Chem.,* **50**, 53 (2002).

176. S.M. Dagher, Z.K. Hawi, and N.S. Kawar, *J. Environ. Sci. Health, Part B,* **34**, 849 (1999).
177. A. Abad, J.J. Manclus, F. Mojarrad, J.V. Mercader, M.A. Miranda, J. Primo, V. Guardiola, and A. Montoya, *J. Agric. Food Chem.,* **45**, 3694 (1997).
178. G. Giraudi, C. Baggiani, A. Cosmaro, E. Santia, and A. Vanni, *Fresenius' J. Anal. Chem.,* **360**, 235 (1998).
179. B. Mickova, L. Karasova, L. Fukal, I. Hochel, P. Rauch, P. Gregor, J. Hajslova, F. Fini, S. Girotti, A. Abad, J.J. Manclus, J.V. Mercader, and A. Montoya, *Czech J. Food Sci.,* **18**, 266 (2000).
180. S. Miyake, A. Hayashi, T. Kumeta, K. Kitajima, H. Kita, and H. Ohkawa, *Biosci. Biotechnol. Biochem.,* **62**, 1001 (1998).
181. V. Issert, R. Lazaro, F. Lamaty, V. Rolland, P. Besancon, B. Caporiccio, P. Grenier, and V. Bellon-Maurel, *Amino Acids,* **17**, 377 (1999).
182. M. Lee, D.R. Walt, and P. Nugent, *J. Agric. Food Chem.,* **47**, 2766 (1999).
183. D.L. Young, C.A. Mihaliak, S.D. West, K.A. Hanselman, R.A. Collins, A.M. Phillips, and C.K. Robb, *J. Agric. Food Chem.,* **48**, 5146 (2000).
184. K. Li and Q.X. Li, *J. Agric. Food Chem.,* **48**, 3378 (2000).
185. K. Hemalatha, N.B.K. Venugopal, and B.S. Rao, *J. AOAC Int.,* **84**, 1001 (2001).
186. S. Miyake, K. Morimune, Y. Yamaguchi, K. Ohde, M. Kawata, S. Takewaki, and Y. Yuasa, *J. Pestic. Sci.,* **25**, 10 (2000).
187. M.J. Moreno, A. Abad, and A. Montoya, *J. Agric. Food Chem.,* **49**, 72 (2001).
188. A. Szekacs and B.D. Hammock, *J. Agric. Food Chem.,* **43**, 2083 (1995).
189. A.R. Fernandez-Alba, A. Valverde, A. Agueera, M. Contreras, and D. Rodriguez, *Anal. Chim. Acta,* **311**, 371 (1995).
190. G. Giraudi, I. Rosso, C. Baggiani, C. Giovannoli, A. Vanni, and G. Grassi, *Anal. Chim. Acta,* **392**, 85 (1999).
191. F. Gueguen, F. Boisde, A.-L. Queffelec, J.-P. Haelters, D. Thouvenot, B. Corbel, and P. Nodet, *J. Agric. Food Chem.,* **48**, 4492 (2000).
192. A.L. Queffelec, F. Boisde, J.P. Larue, J.P. Haelters, B. Corbel, D. Thouvenot, and P. Nodet, *J. Agric. Food Chem.,* **49**, 1675 (2001).
193. J.M. Yeung and W.H. Newsome, *Bull. Environ. Contam. Toxicol.,* **54**, 444 (1995).
194. T.S. Lawruk, A.M. Gueco, S.W. Jourdan, A.M. Scutellaro, J.R. Fleeker, D.P. Herzog, and F.M. Rubio, *J. Agric. Food Chem.,* **43**, 1413 (1995).
195. C. Jahn and W. Schwack, *J. Agric. Food Chem.,* **49**, 1233 (2001).
196. C. Danks, M.Q. Chaudhry, L. Parker, I. Barker, and J.N. Banks, *Food Agric. Immunol.,* **13**, 151 (2001).
197. S. Cairoli, A. Arnoldi, and S. Pagani, *J. Agric. Food Chem.,* **44**, 3849 (1996).
198. A. Abad, J.J. Manclus, M.J. Moreno, and A. Montoya, *J. AOAC Int.,* **84**, 156 (2001).
199. D.L. Brandon, R.G. Binder, A.H. Bates, and W.C.J. Montague, *Food Agric. Immunol.,* **7**, 99 (1995).
200. R.J. Bushway, B.E. Young, L.R. Paradis, and L.B. Perkins, *J. AOAC Int.,* **77**, 1243 (1994).
201. E. Watanabe, S. Watanabe, S. Ito, M. Hayashi, T. Watanabe, Y. Yuasa, and H. Nakazawa, *J. Agric. Food Chem.,* **48**, 5124 (2000).
202. A. Szekacs, A. Cairoli, H.M. Le, and S. Pagani, *Acta Phytopathol. Entomol. Hung.,* **31**, 293 (1996).
203. T. Chen, C. Dwyre-Gygax, S.T. Hadfield, C. Willetts, and C. Breuil, *J. Agric. Food Chem.,* **44**, 1352 (1996).
204. T. Chen, C. Dwyre-Gygax, R.S. Smith, and C. Breuil, *J. Agric. Food Chem.,* **43**, 1400 (1995).
205. R.J. Bushway, T.S. Fan, B.E.S. Young, L.R. Paradis, and L.B. Perkins, *J. Agric. Food Chem.,* **45**, 1138 (1994).
206. R.J. Bushway, B.E. Young, L.R. Paradis, L.B. Perkins, S.K. Martin, and M.P. Brown, *J. AOAC Int.,* **77**, 1243 (1994).
207. J.A. Itak, M.Y. Selisker, D.P. Herzog, J.R. Fleeker, E.R. Bogus, and R.O. Mumma, *J. AOAC Int.,* **77**, 86 (1994).

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Immunologically based assays for pesticide/veterinary medicine residues in animal products

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1 Introduction

Immunoassay (IA) methods are effective for residue detection in a wide variety of media. Emerging immunoassay applications have increased dramatically over the past decade. Many books,[1–3] monographs,[4,5] and review chapters[6–10] have recently dealt with immunoassay techniques. Advantages of immunoassays relative to instrumental methods of analysis include mobility, potential for high sample throughput, modest cost per sample, simple sample cleanup, low solvent use, and the ease of data interpretation. These advantages are particularly relevant to the analysis of pesticide and veterinary medicine residues in food animal products that require rapid, reliable results for large numbers of samples. Immunoassays of xenobiotic residues in livestock products are technically demanding. The diverse nature of matrices, the high sensitivities required for most applications, and the complex pharmacokinetic relationship between the target analyte and its metabolites might complicate assay development. The purpose of this article is to describe the use of immunoassay techniques to detect xenobiotic residues in eggs, milk, and meat from food animals.

2 Immunoassays and animal production agriculture

Immunoassays have had two main applications in animal agriculture. Qualitative immunoassays are used as screening tools to determine whether an animal, or a group of animals, have been exposed to a compound of interest. Regulatory agencies are required to screen a large numbers of samples for violative residues in live animals or in animal carcasses. For example, Kuiper *et al*.[11] reported that approximately 30 000 animals in the European Union were analyzed for β-agonists annually. Illegal residues can be detected by qualitative immunoassays, with subsequent confirmatory or determinative assays.[11–13] The practical use of qualitative immunoassays may be limited by inappropriate limits of detection, extensive cross-reactivities, and a high incidence of

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false positives. As discussed below, extensive cross-reactivity within a class of compounds may be beneficial for a qualitative assay designed to simultaneously screen many drugs within a class (e.g., steroids, β-agonists, sulfonamides).

Quantitative immunoassays have been used extensively for the determination of endogenous hormones and animal health drugs for use in pharmacokinetic, residue, or physiological studies. For example, estrogens used in anabolic implants were quantified by immunoassay[14,15] in support of registration. Furthermore, studies investigating the physiological levels of steroid hormones also used immunological techniques.[16] Quantitative immunoassays have also been used as screening devices to determine whether drug residues exceed established maximum residue limits (MRLs) or tolerances in edible tissues.[17–19] For these applications, a 'cut-off' value is set at the tolerance or MRL; samples detected above this level are 'positive', and samples below this level are 'negative.'

3 Considerations involved in immunoassay development

Several considerations influence the suitability of the immunoassay as a qualitative or quantitative tool for the determination of tissue residues. These include the assay format, the end user (on-farm or laboratory use), effects of sample matrix on the analysis, cross-reactivity considerations, detection levels required of the assay, target tissues to be used in the assay, and the use of incurred or fortified tissues for validation of the immunoassay against accepted instrumental methods. Although these variables are often interrelated, each topic will be discussed in further detail below.

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3.1 Immunoassay format

Direct and indirect competition formats, illustrated in Figure 1, are widely used for both qualitative and quantitative immunoassays. Direct competition immunoassays employ wells, tubes, beads, or membranes (supports) on to which antibodies have been coated and in which proteins such as bovine serum albumin, fish gelatin, or powdered milk have blocked nonspecific binding sites. Solutions containing analyte (test solution) and an analyte–enzyme conjugate are added, and the analyte and antibody are allowed to compete for the antibody binding sites. The system is washed, and enzyme substrates that are converted to a chromophore or fluorophore by the enzyme–tracer complex are added. Subsequent color or fluorescence development is inversely proportionate to the analyte concentration in the test solution. For this assay format, the proper orientation of the coated antibody is important, and anti-host IgG or protein A or protein G has been utilized to orient the antibody. Immunoassays developed for commercial purposes generally employ direct competition formats because of their simplicity and short assay times. The price for simplicity and short assay time is more complex development needed for a satisfactory incorporation of the label into the antibody or analyte without loss of sensitivity.

For indirect immunoassay methods, the antigen (analyte) is bound to support materials and excess binding sites are blocked. Analyte and primary antibody are then added simultaneously, followed by the addition of enzyme-labeled secondary antibody and color reagent. The bound analyte (coating antigen) and free analyte (in

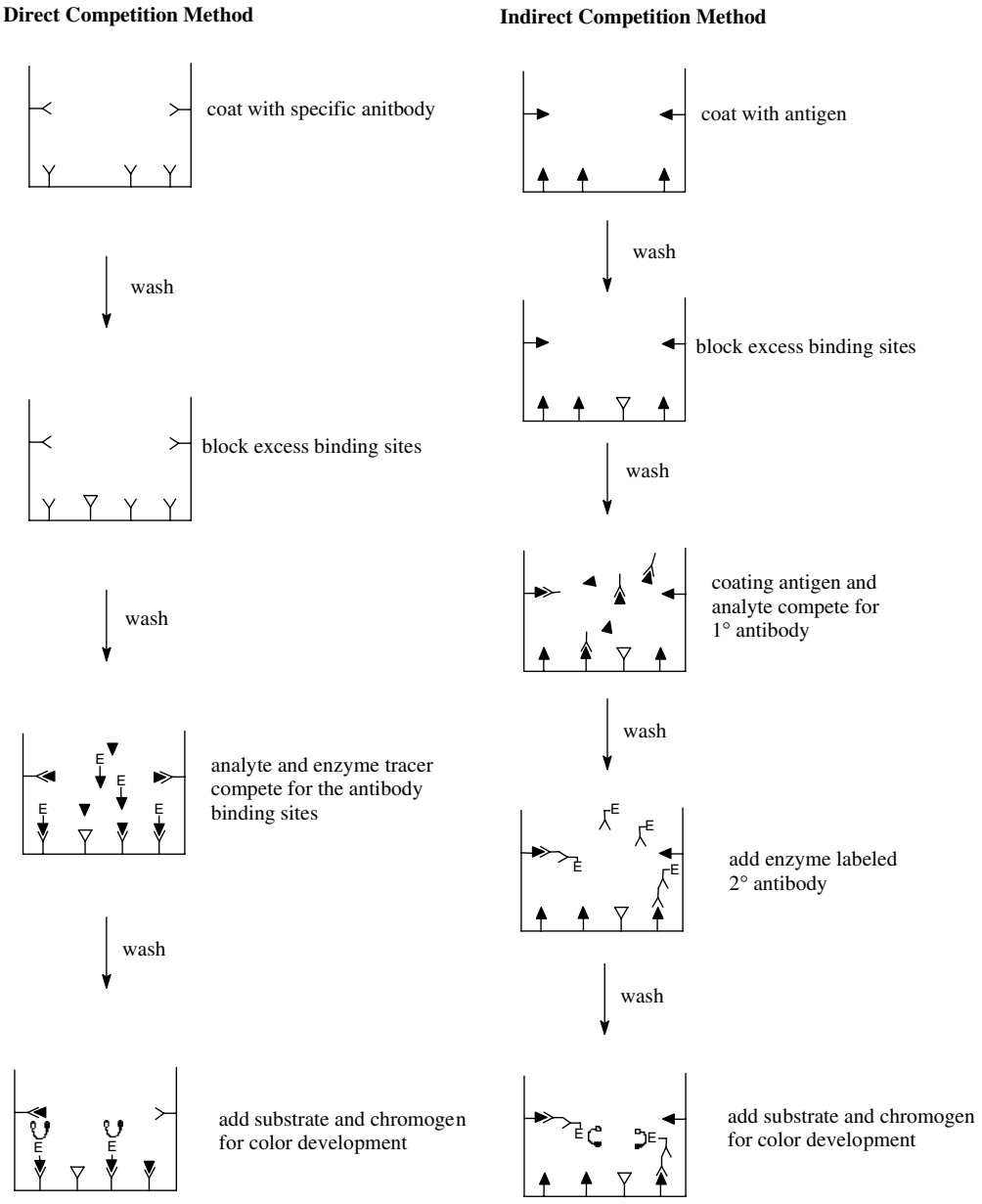


Figure 1 Schematic sequence of the direct and indirect competitive ELISA. The principle difference is that for direct competitive immunoassay, the well is coated with primary antibody directly, and for indirect competitive immunoassay, the well is coated with antigen. Primary antibody (⋎), blocking protein (▽), analyte (▼), analyte–tracer (E▼), enzyme labeled secondary antibody (⋏E), color development (ᔓ)

the test solution) compete for the primary antibody, the end result being that color development is inversely proportional to analyte concentration. In general, indirect immunoassays require that the primary and secondary antibodies be added separately and require longer incubation times than direct competition assays. Enzyme-labeled secondary antibodies are generally commercially available and, as a result, laboratories that develop their own immunoassays commonly develop indirect competition assays. These assays are often more sensitive since multiple secondary antibody molecules may bind to the primary antibody enhancing the signal. Even greater sensitivity may be achieved using a biotinated primary antibody that binds extremely tightly with avidin/streptavidin preparation.

3.2 End user

One important criterion during the development of the immunoassay is to consider the needs of the end user. For immunoassays to be used in the laboratory for the quantitative determination of a drug, long incubation times, extensive wash procedures, and sensitive detection equipment may be required. However, for immunoassays that are to be used in the field (screening assays), simplicity, timeliness, ease of interpretation, and a low incidence of false negatives are important requirements. For a given analyte, both simple and more complex formats may be appropriate. For example, the global misuse of the β-adrenergic agonist clenbuterol has resulted in several immunoassays developed for field use,[20,21] quantitative screens for the laboratory,[22–24] and highly validated quantitative assays to be used in the laboratory.[25–27]

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3.3 Assay interferences

The analytical response generated by an immunoassay is caused by the interaction of the analyte with the antibody. Although immunoassays have greater specificity than many other analytical procedures, they are also subject to significant interference problems. Interference is defined as any alteration in the assay signal different from the signal produced by the assay under standard conditions. Specific (cross-reactivity) and nonspecific (matrix) interferences may be major sources of immunoassay error and should be controlled to the greatest extent possible. Because of their different impacts on analyses, different approaches to minimize matrix effects and antibody cross-reactivity will be discussed separately.

3.3.1 Matrix effects

Nonspecific interferences are associated mainly with the solvent environment of the immunoassay. Variables such as pH, ionic strength, protein or lipid concentration, endogenous enzymes, and(or) the presence of chromophores may dramatically affect the signal generated by the analyte–antibody interaction. These variables are affected by sample preparation, tissue type, animal species, physiological state, disease states, and even feeding conditions. To measure matrix effects, assay results of calibration curves prepared in buffer or water are compared with those prepared in the test matrix (Figure 2). A change in the calibration curve prepared in matrix (or matrix extract),

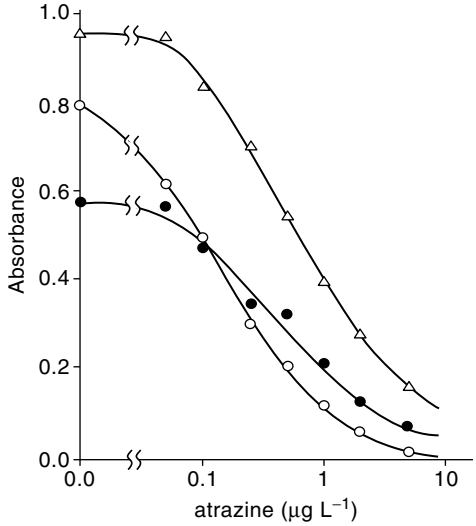


Figure 2 An illustration of matrix effects on immunoassay performance. Calibration curves of atrazine were run in buffer (●), in skim milk (○) and in whole milk (△). Reprinted from M. Franek, V. Kolar and S. A. Evemin, *Analytica Chimica Acta*, **311**, 349–356, Copyright 1995, with permission from Excerpta Medica Inc

relative to the calibration curve prepared in water or buffer, indicates that a matrix effect has occurred. Matrix effects may be quantified by changes in the parameters of the curve fit (B_0, IC_{50}, and slope). Matrix effects may cause significant quantitative errors and, if severe, can render the assay useless. Methods to detect and eliminate matrix effects in agrochemical immunoassays in plants have been discussed by Skerritt and Amita Rani,[28] but their discussion is also appropriate for immunoassays of animal samples.

Two general approaches have been used to overcome matrix effects: (1) partial purification of the analyte prior to analysis by immunoassay ('cleanup' methods) and (2) the use of a matrix blank when preparing the calibration curve. Both options are widely used, but each has its individual limitations.

Purification of the analyte for most immunoassay applications does not require the rigorous multi-step purification procedure that instrumental analyses may require. Multiple filtration, solvent extraction, or solid-phase extraction (SPE) can eliminate the sample matrix effect. In general, tissue samples are homogenized followed by centrifugation; the supernatant, which contains the analyte, is generally recovered and used for the assay. Further purification of the analyte by filtration, SPE, or solvent extraction may be necessary to eliminate the matrix effect of the tissue homogenate. Although these steps are easily accomplished in a laboratory setting, purification steps may not be appropriate for field tests.

The use of a matrix blank is the simplest way to overcome a matrix effect, but the analyst must ensure that the matrix blank is uniform and does not change between sample sets. Acquiring a uniform blank matrix may be problematic if assays are conducted over an extended time period. Caution must be taken when an analysis, validated for one matrix or species, is used for a different tissue or species, because

interferences within a matrix blank may be species or tissue specific. Even within a species, animal health, dietary differences, individual animal differences, and a host of other factors may change the chemical milieu of which target matrices are composed. For example, in ruminants, dietary changes may influence urinary pH,[29] which could influence the ionization of the analyte that may alter binding to antibody. Alternatively, pH changes could alter the ionization of an interfering compound causing an increase or decrease of assay sensitivity. Haasnoot *et al.*[22,30] noted that the matrix effects differ between calf and cow urine for β-agonist immunoassays. Presumably, this difference in matrix effect of bovine urine is due to urine composition differences between ruminating (cows) and nonruminating (calves) animals. Because matrix effects depend upon the bulk properties of the solution containing the analyte, the analyst should be aware of the properties of the matrix that is being analyzed. This knowledge will allow the analyst to approach the processing of the sample in a logical fashion.

3.3.2 Cross-reactivity

Cross-reactivity is defined as the ratio of the IC_{50} of the interfering substance to that of the analyte, expressed as a percentage. The IC_{50} can be easily and accurately calculated from competition curves using widely available computer software. In general, cross-reactivity occurs with compounds that share structural similarities to the portion of the analyte that binds to the antibody. Once bound, the antibody–ligand interaction causes a detectable assay response in a manner similar to the target analyte. Compounds within a structural class (e.g., estrogens, androgens, β-agonists) may share common structural elements and could exhibit varying degrees of cross-reactivity. More generally, any structural similarity of a chemical to the epitope of the analyte may cause cross-reactivity to the antibody. Specific interference is measured as cross-reactivity. Examination of a sufficient number of compounds within a structural class will establish the type of structures that may cause analytical problems.

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Because of the structural similarity of metabolites to their parent drug, they may strongly cross-react in immunoassays targeted towards the parent drug. This is only true, however, if the site of metabolism is not part of the epitope that the antibody recognizes. Several examples of cross-reactivity relevant to animal agriculture exist. Yamomoto and Iwata developed an antibody towards clenbuterol and tested its cross-reactivity towards five clenbuterol metabolites[31] (Met-1–5 in Table 1). None of the metabolites had a cross-reactivity of greater than about 1%. The antibody generated by Yamamoto and Iwata was prepared by conjugating human serum albumin to the diazotized aromatic amine of clenbuterol. The exposed *tert*-butylaminoethanoamine region (see Table 1 for structure) became an important region for antibody binding. Hydroxylation of the *tert*-butyl group (Met-5) resulted in a 99% loss in affinity to the antibody. In a study designed to investigate the affinity of several commercial clenbuterol kits to metabolites of clenbuterol, Shelver and Smith[32] demonstrated that clenbuterol sulfamate metabolites cross-reacted significantly with commercial clenbuterol kits. Similar to the work of Yamamoto and Iwata, the metabolites resulting from the hydroxylation of the *tert*-butyl group of clenbuterol only have very low cross-reactivity in these commercial kits. The difference in cross-reactivity to the oxidized metabolites and the sulfamate metabolite is that the sulfur conjugation site is at the aromatic amine used for diazotization during the antigen building process. For these

Table 1 Cross-reactivity of clenbuterol metabolites with various immunoasssays

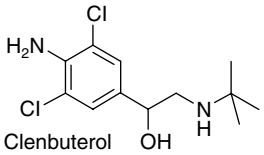
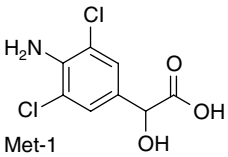
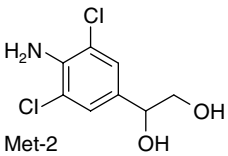
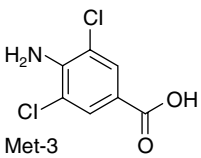
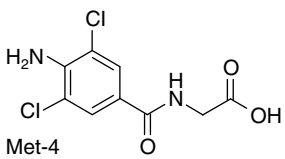
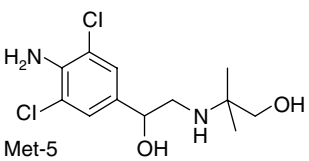
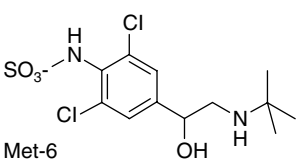
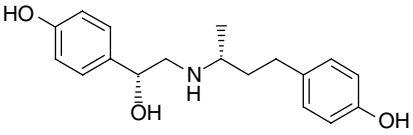
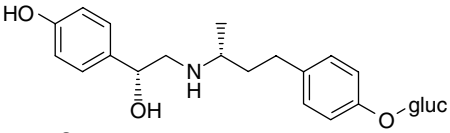
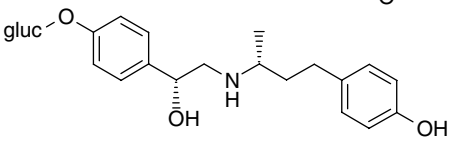
Structure	Immunoassay source	Cross-reactivity (%)
 Clenbuterol	Yamamoto and Iwata[31]	100
	Shelver and Smith[32]	
	IA Kit A	100
	IA Kit B	100
	IA Kit C	100
 Met-1	Yamamoto and Iwata[31]	0
 Met-2	Yamamoto and Iwata[31]	0
 Met-3	Yamamoto and Iwata[31]	0
 Met-4	Yamamoto and Iwata[31]	0
	Shelver and Smith[32]	
	IA Kit A	0.3
	IA Kit B	0.2
	IA Kit C	0.2
 Met-5	Yamamoto and Iwata[31]	<1%
 Met-6	Shelver and Smith[32]	
	IA Kit A	72
	IA Kit B	77
	IA Kit C	42
Clenbuterol glucuronides	Shelver and Smith[32]	
	IA Kit A	0.2–1.6
	IA Kit B	0.2–1.1
	IA Kit C	0.1–0.6

Table 2 Cross-reactivity of racemic ractopamine and ractopamine glucuronide metabolites to a monoclonal antibody developed against racemic ractopamine

Parent ractopamine			
Structure		Stereochemical composition	Cross-reactivity (%)[a]
		*RR*, *RS*, *SR*, *SS*	100
		RR	489
		SR	134
		SS	1.9
		RS	0.9
Ractopamine glucuronides			
Structure	Fraction[b]	Stereochemical composition	Cross-reactivity (%)[a]
	A	*RS*, *SR*	1.0
	B	*RR*, *SS*	3.1
	C	*RR*, *RS*, *SR*, *SS*	384

[a]Percentage cross-reactivity is defined as the ratio of the IC_{50} of the testing substance (ractopamine stereoisomer or metabolite) and the IC_{50} of the ractopamine racemic mixture, expressed as a percentage.
[b]Chromatographic fractions, glucuronide fractions A, B, and C were separated by reversed-phase chromatography.

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antibodies, only the presence of clenbuterol sulfamate would result in significant immunoassay errors. For most quantitative clenbuterol assays, cross-reactivity to oxidized metabolites does not represent a problem because of the low cross-reactivity to these metabolites.[31] In addition, because sulfamate metabolites are not present in great quantities in cattle liver or urine,[33] this metabolite may not appreciably alter quantitative results in these matrices.

In contrast, glucuronide conjugates of ractopamine, another β-adrenergic agonist, make up the majority of the total tissue residues of ractopamine.[34] A monoclonal antibody developed by Shelver *et al.*[35] exhibited equal cross-reactivities to major ractopamine metabolites and the parent drug (Table 2). The qualitative value of such an immunoassay is readily apparent if one wanted to determine whether or not animals were exposed to ractopamine. Quantitative analysis using the immunoassay might be more difficult in the absence of steps to separate glucuronide conjugates from parent ractopamine.

For the ractopamine antibody, the pattern of cross-reactivity shown in Table 2 strongly suggests that the epitope resides on the *p*-hydroxyl group of the phenyl ring of the *N*-butyl group of ractopamine in the *R* configuration. Glucuronide conjugates

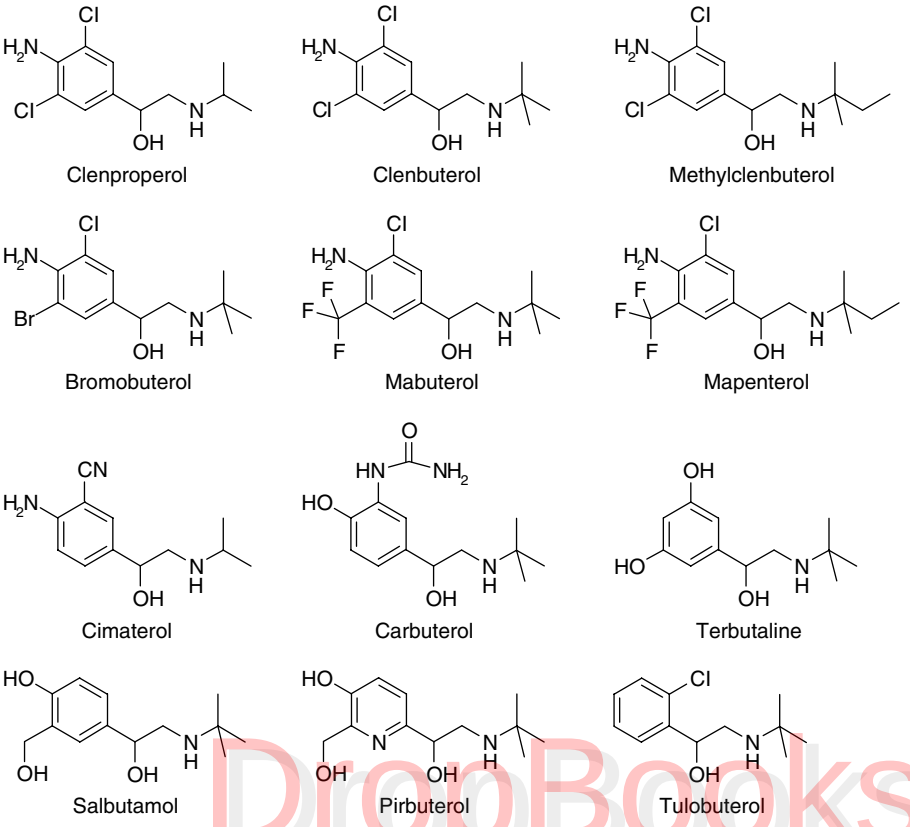
A and B, which are conjugated to the *p*-hydroxyl group of this phenyl ring, had cross-reactivities of only about 1–3%; in contrast, glucuronides (C) conjugated to the *p*-hydroxyl group of the phenyl ring of the *N*-hydroxylethyl group had cross-reactivities of 384% relative to racemic ractopamine. These results indicate that when the epitopic region of the parent drug is unaltered by metabolism, high cross-reactivities will likely occur. Conversely, metabolic alterations of the epitope region usually cause low cross-reactivities.

The ultimate impact of cross-reactivity to metabolites depends upon the purpose of the assay. For assays in which the goal is to determine whether an animal has been exposed to a drug, high cross-reactivity to metabolites is a benefit. Such scenarios currently exist for meat products produced in the USA where ractopamine has been approved and exported to Europe, where the use of hormones and β-agonists is banned. An antibody that detected both ractopamine and ractopamine glucuronide would be of great value in determining animal exposure. In contrast, if the assay were quantitative and a tolerance or a maximum residue level needed to be assessed, having an immunoassay specific for a marker compound for which threshold levels exist would be best. For immunoassays used for quantitative purposes, cross-reactivity with metabolites will overestimate the actual analyte residue.

As indicated above, the use of β-agonists to increase carcass leanness is illegal in Europe; nevertheless, illegal use of this drug class has been extensive[11] and subsequent surveillance efforts have been expansive.[36,37] A limitation of monitoring efforts has been the specificity of the screening assays available for use. Figure 3 shows many of the β-agonists thought to have the potential for illegal use in livestock.[20,38–40] All phenethanolamine β-adrenergic agonists are comprised of a substituted phenyl group, an ethanolamine 'backbone,' and an *N*-alkyl group. For clenbuterol and many of the β-agonists shown in Figure 3, a *tert*-butyl group serves as the *N*-substituent. Several groups have attempted to use the substituent similarity in the *N*-alkyl group among β-agonists as a basis to form multi-residue screening assays. For example, Vanoosthuyze *et al.*[21] built an antibody against salbutamol by conjugating it to ovalbumin with succinic anhydride. The antibody to salbutamol had cross-reactivities of 9–56% for β-agonists having a *tert*-butyl substituent [clenbuterol (56%), mabuterol (44.3%), bromobuterol (31%), and terbutaline (9%)] but only about 2% for compounds having an *N*-isopropyl substituent (clenproperol and cimaterol). Haasnoot *et al.*[22] built an antibody against clenbuterol after diazotization and conjugation to bovine serum albumin. For the antibody produced against clenbuterol, cross-reactivities were high for only clenbuterol and mabuterol and were low ($<10\%$) for other compounds containing an *N*-*tert*-butyl substituent. To date, a 'universal' antibody has not been built that has broad cross-reactivity to β-agonists sharing an identical *N*-alkyl substituent. Because of the thousands of structural variations that can be made to the phenethanolamine backbone, screens that have extensive cross-reactivity are needed.

3.4 Detection levels (sensitivity)

Adequate sensitivity is required to measure an analyte accurately at the MRL (or tolerance) set by the regulatory agency. Tolerances and MRLs sometimes differ between agencies and may change as new scientific evidence indicates that the residue level



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Figure 3 Structures of phenethanolamine β-adrenergic agonists of potential human food safety concern. Multi-residue methods have been developed with the goal of broad cross-reactivity so that simultaneous screening may occur; efforts have not been particularly successful

of a given compound represents a greater or lesser risk. Comparisons of some MRLs set in the European Union (EU) and tolerances established by the US Food and Drug Administration (FDA) are shown in Table 3. Most authors report immunoassay sensitivity as the limit of detection (LOD), which can be defined as either the concentration giving the mean absorbance of the blank plus three standard deviations or as a percentage of B/B_0 (e.g., 90% B/B_0), where B_0 is the value of the absorbance with no analyte and B is the value of the absorbance in the presence of analyte. A value related to LOD is the blank plus six standard deviations, which is known as the limit of quantitation (LOQ); similar to the LOD, the LOQ may be defined as a percentage of B/B_0 (e.g., 80% B/B_0). Assessing whether a residue is present in a sample when the analytical results of the immunoassay are close to the LOD is risky because high numbers of false positives and false negatives could be encountered. Sawaya *et al.* [41] reported an example of such evidence after a commercial kit was used to screen sheep samples; they found that 70% of the samples were false positive when a cutoff equal to the LOD (0.5 $\mu g\,kg^{-1}$) was used. A small percentage of false positives are tolerable if a confirmatory method can be used, but for food analysis false negatives are less acceptable, because a false negative sample would expose the public to contaminated food.

Table 3 Examples of therapeutic agent maximum residue limits (MRLs) (mg kg^{-1}) from EU[a] and tolerance levels (TLs) (mg kg^{-1}) from US FDA[b]

Compound [CAS No.]	TL (US FDA)	Species[c]	Matrices[c]	MRL (EU)	Species[c]	Matrices[c]
Amoxicillin [26787-78-0]	0.01	B	Edible tissues	0.05	n.s[d]	M, L, K, F
	0.01	B	Mi	0.004	B, O	Mi
Ampicillin [69-53-4]	0.01	B, P	Edible tissues	0.05	B, P	M, L, K, F
	0.01	B	Mi	0.004	B, O	Mi
Cloxacilllin [61-72-3]	0.01	B, O	Edible tissues	0.3	n.s	M, L, K, F
	0.01	B	Mi	0.03	B. O	Mi
Penicillin [61-33-6]	0.05	B	Edible tissues			
	0	Ch, G, Q, P, Ca	Eggs, Mi, Milk products			
	0.01	T	Edible tissues			
Chloramphenicol [56-75-7]				0.01	n.s	M, L, K, F
Erythromycin [114-07-8]	0.1	B, P	Edible tissues	0.4	B, O, P, Ch	L, K, M, F
	0	B	Mi	0.04	B, O	Mi
	0.025	Ch	Eggs	0.2	Ch	Eggs
	0.125	Ch, T	Edible tissues			
Gentamicin [1403-66-3]	0.1	T	Edible tissues			
	0.1	P	M			
	0.3		L			
	0.4		F, K			
Streptomycin [57-92-1]	2	Ch, P, B	K			
	0.5		Other edible tissues			
Sulfamethazine [57-68-1]	0.1	Ch, T, B, P	Edible tissues, Mi	0.1	n.s	M, L, K, F, Mi
Oxytetracycline [79-57-2]	3	T, Ch	K	0.6	n.s	K
	1	T, Ch	M, L, F, Skin			
				0.3	n.s	L
				0.2	n.s	E
	0.1	P, Ca, S	Edible tissues	0.1	n.s	M, Mi
				0.01	n.s	F
Tetracycline [60-54-8]	0.25	B, P, C, Ch, T	Edible tissues			
Trimethoprim [738-70-5]				0.05	n.s	M, L, K, F, Mi
Enrofloxacin [93106-60-6]				0.03	B, P, Ch	M, L, K
Monesin [17090-79-8]	0.05	B, O	Edible tissues			
	1.5	Ch, T, Q	M			
	3		Skin, F			
	4.5		L			
Sarafloxacin [98105-99-8]	No requirement for edible turkey and broiler chicken tissues			0.1	Ch	L
				0.01		F with skin
Halofunginone [352464-99-4]	0.1	Ch, T	M			
	0.3		L			
	0.2		Skin, F			
Levamisole [14769-73-4]	0.1	B, Ca, P	Edible tissues	0.01	n.s	M, K, L, F, Mi

[a]Data obtained from ref. 111.
[b]Data obtained from ref. 112.
[c]B = bovine; Ca = caprine; Ch = poultry; E = equine; G = gamebirds; O = ovine; P = porcine; Q = quail; S = salmonids, catfish, and lobsters; T = turkey; M = muscle; Mi = milk; L = liver; K = kidney; E = eggs; F = fat.
[d]Not specified.

Assessment and definition of sensitivity are often described for quantitative analysis but are of equal importance for qualitative devices of the dip-stick type that are very popular for farm- or field-based screening assays. Because of the somewhat subjective nature of visually assessed assays, the assay's sensitivity must be validated using a number of observers to determine at what level a test is deemed positive. The number of false positives and false negatives must be carefully determined in order to balance consumer safety and potential economic loss to animal producers.

3.5 Target tissues

The distribution and metabolism patterns of the target xenobiotic profoundly affect selection of the target tissue and the parent compound or a metabolite as the marker compound. For example, a clenbuterol is concentrated in the retina as much as 100-fold relative to other tissues.[42,43] This makes the retina an extremely sensitive and valuable tissue for monitoring the illegal use of this drug.[13] For drugs that have been approved for use in livestock species and for which a tolerance exists, the target tissue will coincide with the statutory target tissue designated by the FDA or EU. In these cases, a metabolite may predominate in the edible tissue. Nevertheless, the target levels of the immunoassay should coincide with the residue level of the marker compound, because tolerances are set on the basis of the marker compound.[44] In order to validate fully an immunoassay for an animal health drug, metabolism studies will be necessary to determine the toxicity of metabolites and the persistence and concentration of metabolites and to identify target tissues. For widely used pharmaceuticals, metabolism studies may be available in the open literature, but for newer or less frequently used compounds the data may need to be generated.

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3.6 Assay validation using incurred or fortified tissues

An important consideration during the validation process of immunoassay is whether to use samples with incurred analyte or to use fortified samples. Performing recovery studies with fortified samples is easier and provides an assessment of the precision and accuracy of the analytical data, but analyte extraction from fortified samples may not accurately mimic the extraction efficiencies of analyte from incurred samples. Incurred samples are prepared by treating target animals with the test drug using the conventional route of administration and collecting the edible tissues at appropriate times. The residue which remains in the target tissue is termed 'incurred.' The use of incurred residue for the validation of an immunoassay requires confirmation by a validated instrumental method, because the amount of analyte in the incurred tissue is unknown. Results from the validated instrumental analytical method are compared with the immunoassay being validated. For a valid comparison, the sample preparation methods must be the same for both assays. Immunoassays should generally be validated using incurred samples, because the analyte and metabolites can be distinguished in a realistic manner. A good correlation of the analytical values between the immunoassay and the validating method indicates that they are measuring the same analyte. However, a poor correlation is frequently found if the immunoassay method cross-reacts extensively with metabolites present in the sample.

4 General sample treatments for eggs, milk, and meat

One advantage of using an immunoassay technique is its compatibility with the aqueous phase. Immunoassays are very convenient and have often been used for residue monitoring of aqueous samples such as water, juice, urine, serum, and plasma. Fewer examples of immunoassay use in eggs, milk, and meat are found in the open literature. Although immunoassays generally tolerate interferences better than the instrumental methods, matrix effects may occur without proper tissue preparation and ultimately result in error. For the analysis of chemical residues in various matrices, an analyst faces the dual problems of optimizing the analysis (sensitivity, precision, accuracy, specific interferences) and minimizing the detrimental effects of the matrix (nonspecific interferences). Knowledge of the physicochemical properties of the matrix will expedite a rational development strategy. Aerts *et al.* [45] detailed analytical strategies for veterinary drugs and residues in edible products. The focus of our discussion is on the matrix effects on immunoassay, whereas Aerts *et al.* focused on these effects on instrumental analysis.

4.1 Eggs

Residue determination in eggs is most commonly associated with chickens, although other species may be of interest for agrochemicals that are widely dispersed in the environment. Chicken eggs contain two physically distinguishable components, yolk (~32% of the total egg mass) and albumen (~56% of the total egg mass). The major components of egg yolks are water (~50% of yolk mass), lipid (>30% of yolk mass), including triglycerides, phospholipids, and cholesterol, and protein (~16% of yolk mass). In contrast, egg albumen contains very little lipid (<0.01%), but albumen contains substantial protein (~10% of albumen mass). Egg albumen is essentially a colloidal suspension of glycoprotein in water. Because of the great differences in lipid composition between egg albumen and yolk, xenobiotic residues may distribute to yolk or albumin based on the lipophilic nature of the xenobiotic.[45] Such a distribution has not been observed for a number of drugs, however, and the ultimate destination of the drug residue is likely determined by specific protein interactions in addition to lipophilic partitioning.[46]

The complex chemical and physical structure of eggs makes immunochemical analysis difficult without sample cleanup. Simple dilution of the egg mass will not be appropriate for most immunoassays because of the relatively high lipid and protein content of eggs. Whether the egg will be analyzed intact or as the yolk and(or) albumen will depend on the study objective. Tolerances of animal health drugs are determined based on the content of the whole egg, so combining the albumen and yolk to analyze the residues in whole egg may be appropriate for some studies. Homogenization and extraction often lead to emulsions caused by the presence of natural emulsifiers in the egg. Analyses of residues in eggs will likely require solvent extraction followed by centrifugation. The centrifugation requirement may limit the development of field-based assays where access to a centrifuge is unlikely. Arnold and Somogyi[47] found that removing lipid from yolk, or from yolk and albumen processed together, was required prior to conducting a radioimmunoassay of chloramphenicol

residue. These authors homogenized eggs in acetonitrile, centrifuged the samples, and then partitioned lipids into hexane. Chloramphenicol was ultimately extracted from the aqueous layer with ethyl acetate. Owing to the difficulties in the separation of the analyte from interferences present in eggs, relatively few immunoassays have been developed. Improved methods for the preparation of egg samples prior to immunoassay will be required before widespread application of immunoassay to eggs is realized.

4.2 Milk

Because milk is consumed in relatively large amounts, without cooking, and disproportionately by children, a great deal of effort has been expended on measuring drug residues in this matrix. Milk is a complex emulsion in which the oil phase is an intricate mixture of phospholipids, fats, and proteins. The aqueous phase of milk is a colloidal suspension of protein micelles in a solution of various salts, proteins, and carbohydrates. Xenobiotic conjugates are rarely transferred from the systemic circulation into milk,[45] but lactose conjugates of sulfonamide drugs have been found in the milk of treated cows and may be nonenzymatically formed in the mammary gland.[48] Binding of analyte to milk protein may also cause analytical complications and increase variability.

Various techniques have been used to simplify immunoassay analytical procedures, increase reproducibility, and increase the accuracy of milk applications. Decreaming is a common first step, because whole milk's high fat content frequently causes analytical problems. Obviously, decreaming requires that the target analyte be lipid insoluble and will be useful only if the matrix effect is due to interference from the components present in the cream. Because of its simplicity, dilution of the milk has frequently been used prior to immunoassay. The degree of dilution needed is variable, but this simple method is often sufficient to reduce matrix effects to acceptable levels. Dilution decreases an assay's sensitivity, but most immunoassays are extremely sensitive and can easily tolerate the reduced sensitivity. Solid-phase extraction or conventional solvent extraction may also be used to eliminate serious interferences depending on the properties of the analyte and the nature of the interference. Validation of extraction efficiencies and careful control of extraction variables are required. Other methods, such as altering the pH, salting, heating, or adding trifluoroacetic acid to precipitate proteins, have also been applied to improve milk immunoassays. Caution should be observed when harsh extraction or sample preparation steps are utilized, because some analytes (such as β-lactams) may be decomposed by these treatments. Measurement of recoveries of fortified blank samples is particularly necessary when decreaming or protein denaturation procedures are employed.

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4.3 Tissues

The most common meats consumed by humans in developed countries are beef, pork, sheep, chicken, turkey, fish, and more rarely goats. Other minor-use species (and tissues, e.g., tripe) are commonly consumed by certain ethnic groups or infrequently

consumed by the general public. Matrices most commonly described in the literature are liver, kidney, muscle, and adipose tissue. Each tissue type presents unique challenges and requires different cleanup procedures. Nonedible tissues such as hair or eyeballs may be used for surveillance or monitoring purposes.

Animal tissues are complex assortments of various cell types held together by an equally complex extracellular matrix. A major task for the analyst is the destruction of the cellular structure within a tissue in order to release the analyte for extraction or analysis. The procedure must be individualized and validated for each analyte in the tissue and species, with appropriate control of variables that might impact the analysis. For example, for some analytes, adipose tissue causes difficulties during sample cleanup; the amount of adipose tissue within a given tissue will vary with animal maturity, physiological state (mature vs growing animals, lactating vs nonlactating, etc.), nutritional status, and health of the animal.

In general, the physical structure of the tissue must be broken down mechanically followed by an extraction procedure, before the sample can be analyzed. Homogenization using blenders, probe homogenizers, cell disrupters, sonicators, or pestle grinders is particularly useful for muscle, liver, and kidney samples. Regardless of the method used for tissue disruption, the pulse, volume of extraction solvent added, and temperature should be validated and standardized in order to ensure reproducible analytical results. During cell disruption, care should be taken to avoid heat build-up in the sample, because the analyte may be heat labile.

Filtration and(or) centrifugation are needed to separate the liquid phase from cellular debris generated during tissue disruption and(or) extraction. Centrifugation may trap the analyte within the pellet, which will then require subsequent pellet washes; this problem may also occur with filtration. Chemically, tissues are composed of multiple proteins, lipids, nucleic acids, trace metals, and carbohydrates. Association of the analyte with cellular components may vary from covalent binding (that renders the analyte nonextractable), to weak ionic, hydrophobic, or physical associations that are easily overcome by extraction. Altering the pH or ionic strength or adding detergents or solvents may increase extraction efficiency. The conditions must maximize the extraction of the analyte but simultaneously minimize both specific and nonspecific interference.

Specific extraction methods are used to prepare the analyte for immunoassay by freeing the analyte from both specific and nonspecific interferences. Supercritical fluid extraction has been used to decrease the amount of solvent waste generated. Solid-phase extraction has gained popularity, and many different supports are available. One promising extraction and concentration method is immunoaffinity chromatography, which will be addressed later.

5 Food-animal immunoassay applications

In the following discussion, the detection of pesticides and veterinary drugs in food animals by immunoassay will be described. Discussion will be organized by compound class, the specific analyte, and, finally, the tissues examined. The general principles described in the first part of this review provide the rationale in the applications described in the following pages.

5.1 Agrochemical residue immunoassay applications

Pesticides, including insecticides, herbicides, and fungicides, are widely used in agriculture, and the potential for these residues to accumulate in food has led to concern for human safety. Pesticide residues may enter food animals from environmental sources or from treated or contaminated feeds. Immunoassay development for pesticides has had major impacts for pesticide registrations, analysis of residues in foods, monitoring environmental contamination, determination of occupational exposure, and integration of pest management.

The most common matrices tested for pesticide residues are water, soil, fruits, and vegetables. Fewer reports exist on applications targeting matrices of animal origin, although immunoassays have been increasingly important in monitoring human pesticide exposure.[49] The high sensitivities of immunoassays make them the methods of choice for monitoring animal exposure, particularly if portability or high assay throughput is required. Table 4 summarizes pesticide immunoassays that have been evaluated in matrices of food-animal origin. Examples of assay development are described below to illustrate the diversified approaches that have been used for pesticide immunoassays.

Because of the possibility that the herbicide alachlor could adulterate food if either poultry or livestock consumed contaminated materials, Lehotay and Miller[50] evaluated three commercial immunoassays in milk and urine samples from a cow dosed with alachlor. They found that milk samples needed to be diluted with appropriate solvents (1 : 2, v/v) to eliminate the matrix effect. One assay kit (selected based on cost) was also evaluated for use with eggs and liver samples from chickens. Egg and liver samples were blended with acetonitrile, filtered, and diluted with water. Linear calibration curves prepared from fortified egg and liver samples were identical

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Table 4 Examples of immunoassays developed for pesticides and their metabolites

Pesticide [CAS No.]	Matrix	Mab/PAb	Support[a]	LOD ($\mu g\,kg^{-1}$)	Reference
Alachlor [15972-60-8]	Meat		MP	1	52
	Milk, eggs, liver		Plates/MP	0.3, 2, 3	50
	Meat			1.1	51
Atrazine [1912-24-9]	Meat		MP	1	52
	Milk	PAb	Tube	0.5	56
Benomyl [17804-35-2]	Meat		MP	5	52
Carbaryl [63-25-2]	Milk	PAb	Plate	100	113
Carbofuran [1563-66-2]	Meat		MP	3	52
Cyclodienes	Meat, milk, fish	Mab	Plate	100	54
2,4-D [94-75-7]	Meat		MP	14	52
DDT [50-29-3]	Milk	PAb		100	62
Diclofop-methyl [51338-27-3]	Milk	PAb	Tube	230	59
Dieldrin [60-57-1]	Meat		Tube		52
Diflubenzuron [35367-38-5]	Milk		Plate	1	114
Paraquat [4685-14-7]	Milk	PAb	Plate	0.1, 2.5	60
Spinosad [168316-95-8]	Milk			3	61
Thiabendazole [148-79-8]	Liver	Mab	Plate	20	102
Triazines	Milk	Both	Plate	0.01	58

[a]MP: magnetic particle; Mab: monoclonal antibody; PAb: polyclonal antibody.

with the calibration curve prepared in buffer, demonstrating that the matrix effects had been satisfactorily eliminated. The authors determined alachlor residue in egg and liver samples in chickens dosed with 1–10 mg kg^{-1} of alachlor. They found that while the assay was able to determine alachlor residue in the egg for up to 24 h, none was detected in the incurred liver samples. Immunoassay results were confirmed by gas chromatography/mass spectrometry (GC/MS), but no direct correlation of the analytical results between the GC/MS and the immunoassay were reported. Use of incurred samples studied over time demonstrated the importance of testing immunoassay procedures under realistic conditions. The nondetection of alachlor residues in liver indicates that either liver is not a suitable target tissue or parent alachlor is not appropriate as the marker compound for the liver tissue.

France and King[51] combined supercritical fluid extraction (SFE) with a commercially available immunoassay to detect alachlor fortified into bovine liver and lard. Alachlor was detected in lard samples at concentrations greater than 10 μg kg^{-1} and in liver at 3.3 μg kg^{-1}. No false positives were observed, demonstrating the potential of combining SFE with immunoassay. Unfortunately, no calibration curves were shown, nor were recovery experiments performed, because the study was for qualitative purposes. This group has reviewed the need to avoid the co-extraction of interfering substances during SFE procedures.[52] Co-extracted lipids may be a problem for some immunoassay applications, but membrane disk filtration of samples may eliminate the problem. Membrane disk filtrations of liver, ground beef, and lard samples extracted by SFE resulted in the immunological detection of fortified alachlor (20 μg kg^{-1}), atrazine (20 μg kg^{-1}), benomyl (100 μg kg^{-1}), and 2,4-D (200 μg kg^{-1}).

Lehotay and Argauer[53] evaluated two commercially available immunoassay kits, one for aldicarb sulfone and the other for carbofuran, to test fortified ground beef, chicken and pig liver, and bovine milk samples. The MRL is 10 μg kg^{-1} for aldicarb and its metabolites in muscle, milk, and meat by-products and is 50 μg kg^{-1} for carbofuran and its metabolites in the same tissues. The matrix effect was explored by comparing standards made up in water with standards prepared in water or acetonitrile extracts of unfortified tissues. Although the matrix effect was high (50% reduction in absorbance), the presence of carbofuran (50 μg kg^{-1}) in ground beef was easily detected. Although extraction by homogenization of samples in acetonitrile resulted in slightly better recoveries, the acetonitrile offered little advantage over simple sample homogenization in water followed by direct immunoassay. However, matrix effects in liver samples were more severe. Carbofuran fortified into chicken liver and cat food at 25 μg kg^{-1} was easily detected with the immunoassay but could not be detected when fortified into pig liver at the same level. This study clearly points out the importance of having adequate blanks and of using proper validation steps for each analysis, because species differences may dramatically influence immunoassay results.

Experiments with aldicarb sulfone in ground beef[53] involved simple extraction with acetonitrile during tissue homogenization and resulted in a definite immunoassay response at the tolerance level of 10 μg kg^{-1}. A moderate, but rather consistent, matrix effect was observed. A more severe matrix effect was observed in bovine milk, blood, and urine. For the liquid matrices, sample dilution was not a satisfactory strategy, because the assay variability increased at lower concentrations, negating any benefit of reducing the matrix effect. This work clearly demonstrated that matrix effects are

important even in qualitative experiments, but the use of appropriate blanks may overcome limitations of stubborn matrices.

Stanker *et al.*[54] developed a monoclonal immunoassay for heptachlor and related cyclodiene insecticides and applied the assay to meat, milk, and fish samples. The antibody cross-reacted with nine cyclodienes (heptachlor, heptachlor epoxide, chlordane, aldrin, endrin, dieldrin, endosulfan, endosulfan sulfate, and toxaphene) with roughly equal affinity, and limited cross-reactivities (<8%) to lindane and kepone were observed. Because these insecticides accumulate in fatty tissues, extraction and specific chromatographic (Florisil) cleanup procedures prior to immunoassay were developed to minimize background interferences. Fortification of beef adipose tissue with 100 $\mu g\,kg^{-1}$ of heptachlor and fish or heavy cream samples with 50 $\mu g\,kg^{-1}$ of heptachlor, with subsequent extraction and cleanup, resulted in an immunoassay response of twice the background. Beef adipose tissue blanks had the highest background, and heavy cream had the lowest. The method worked for aldrin, chlordane, and heptachlor but not for endrin, dieldrin, endosulfan, or heptachlor epoxide, because the latter compounds remained on the Florisil column. A problem encountered for aldrin, chlordane, and heptachlor was that interferences co-eluted with the analytes during sample cleanup. Stanker *et al.*'s study demonstrates the importance of developing cleanup procedures during assay development and validation and thoroughly testing the procedures.[54] This group had previously developed an immunoassay[55] for permethrin in ground beef that gave a linear response from 50–500 $mg\,kg^{-1}$ (tolerance level 150 $\mu g\,kg^{-1}$). The sample preparation prior to immunoassay, however, was very complex; the preparation involved extraction with aqueous acetonitrile, 'freezing-out' of the fat, extraction into hexane, and chromatography prior to immunoassay. Complex pre-assay processing is usually necessary for lipid-soluble analytes.

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Bushway *et al.*[56] used an atrazine immunoassay with an LOD of 0.5 $\mu g\,kg^{-1}$ to determine atrazine in milk, fruit juices, soft drinks, and various fruits and vegetables. In order to obtain a good correlation between results obtained by enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC), the calibration curve used for the ELISA had to be prepared in milk (or matrix of interest for the particular application) rather than buffer. Data (Bushway *et al.*[56]) demonstrate the importance of properly accounting for the matrix effect prior to performing a quantitative analysis. The same group[57] further studied atrazine in processed milks and found that the assay had an LOD of 0.2 $\mu g\,kg^{-1}$ and a linear range between 0.2 and 6.4 $\mu g\,kg^{-1}$. Milk products studied included skim, low-fat, whole, chocolate, evaporated, nonfat dry milk, and half-and-half (heavy cream). Matrix effects correlated roughly with the fat content of the milk product. Dilution decreased the matrix effect but lowered the sensitivity. Franek *et al.*[58] used an immunoassay to determine *s*-triazine in milk samples, and they also found that the matrix effect changed the B_0 value from that of the buffer. Whole milk had a greater deviation of B_0 compared with skim milk, presumably because of the greater fat content. The authors suggested that a matrix blank is required to generate appropriate calibration curves.

Using a simple solvent extraction procedure to minimize matrix effects, a diclofop-methyl immunoassay was developed for milk, a number of edible plant products, and other matrices.[59] Gas chromatography (GC) and liquid scintillation counting (LSC) of a ^{14}C-labeled analyte were used as reference methods to compare with enzyme immunoassay (EIA) results. The methods were well correlated, with comparison of EIA

with GC and ELISA with LSC having r^2 values of greater than 0.98. These authors, however, did not specify which matrix was utilized to test for these correlations.

Van Emon *et al.*[60] developed an immunoassay for paraquat and applied this assay to beef tissue and milk samples. Milk was diluted with a Tween 20–sodium phosphate buffer (pH 7.4), fortified with paraquat, and analyzed directly. Fortified paraquat was detected in milk at less than 1 $\mu g\,kg^{-1}$, a concentration which is considerably below the tolerance level of 10 $\mu g\,kg^{-1}$. Ground beef was extracted with 6 N HCl and sonication. Radiolabeled paraquat was extracted from ground beef with recoveries of 60–70% under these conditions. The correlation coefficient of ELISA and LSC results for the ground beef sample was excellent, with $r^2 = 0.99$, although the slope was 0.86, indicating a significant but reproducible difference between the assays.

Spinosad, an ingredient in several commercial insect control products, is a natural product that is a mixture of several spinosyns. A commercial immunoassay kit[61] was used to measure spinosad in incurred sample residues from milk and beef tissues (lean beef, kidney, and liver). The samples were extracted with either acetonitrile (milk) or acetonitrile–water (tissues) prior to the IA test. The LOD was 3 $\mu g\,kg^{-1}$, and the LOQ was 10 $\mu g\,kg^{-1}$. The assay kit was able to measure several individual spinosyns, some metabolites, and several degradation products. HPLC was utilized as a confirmatory method but required more intensive cleanup procedures. The results of IA and HPLC were in good correlation, although the IA gave slightly higher results. The IA was not used with heavy cream or in fat samples where the spinosad concentrated, possibly because of interference of lipids with antibody binding.

Beasley *et al.*[62] developed a panel of immunoassays to monitor DDT, its metabolites, and structurally related compounds, but they found that milk has a severe effect on the assay performance. They found that when directly utilizing whole milk, color development was completely inhibited. Even when using 1 : 100 dilutions of whole milk, the assay sensitivity was reduced by 90% (based on the IC_{50} shift, not simply the dilution factor). A number of procedures were evaluated to eliminate the interferences from the fat-soluble analytes. However, many of the procedures that removed interferences also removed the analytes. Extraction with a mixture of solvents and the use of similarly processed blank milk to prepare the standards ultimately yielded more accurate results. This article demonstrates the difficulties encountered in analyzing lipid-soluble analytes.

5.2 Detection of veterinary medicine residues

5.2.1 β-Adrenergic agonists

β-Adrenergic agonists have been used in human medicine as bronchodilators for over 40 years. In the early 1980s, β-agonists were found to enhance leanness and growth when included in the diets of growing animals.[63] While several organizations worked on the registration of β-agonists for use in livestock,[64] a number of poisonings occurred in Europe attributable to the illegal use of the β-agonist clenbuterol in cattle.[11,65] Although acute poisoning of humans consuming animal drugs in meat products is rare,[66] these incidents prompted intense research investigating methods of clenbuterol surveillance. Of primary concern was the development of simple, rapid,

inexpensive, and accurate methods that could be used to ensure the safety of thousands of food samples.

Elliott *et al.*[67] utilized a clenbuterol immunoassay to determine clenbuterol residues in cattle tissues and fluids. The LOD was 0.25 $\mu g\,kg^{-1}$ for liver. Animals were dosed with medicated feed (1.6 $\mu g\,kg^{-1}$ per day), and pairs were slaughtered during the medication phase and at 14, 28, and 42 days after withdrawal. Clenbuterol concentrations in liver and retina/choroid samples were confirmed by GC/MS. Correlation coefficients between the ELISA and GC/MS were $r^2 = 0.92$ for retina/choroid samples and $r^2 = 0.96$ for liver samples, although the ELISA results for the liver were close to twice those of GC/MS. The authors explained that the generally higher clenbuterol concentrations provided by the ELISA relative to GC/MS were due to the existence of clenbuterol metabolites not detected by GC/MS. These authors measured clenbuterol accumulation in various tissues and noted that clenbuterol concentrations in the eye were roughly 20 times higher than those in the liver and about 1000 times those in muscle. Furthermore, after a 14-day withdrawal period, detectable clenbuterol concentrations were present only in one traditionally edible tissue (kidney levels were just detectable), but eye tissues contained appreciable clenbuterol concentrations which remained high even after 42 days of withdrawal.

Meyer and Rinke[43] used immunoassay techniques to measure clenbuterol depletion from 21 tissues of calves treated with clenbuterol twice daily for 21 days. They demonstrated that after 14 days of withdrawal, eyes were the only tissue to contain appreciable concentrations of clenbuterol. As a result of this finding, several groups have subsequently investigated the deposition of clenbuterol in ocular tissues of livestock species. Eye tissues are used to screen animals for clenbuterol exposure,[11,65] but the process for eye monitoring is long and tedious. In an effort to simplify the procedure, Nausch and Galley[42] have shown that the corneas of animals, digested by subtilisin in phosphate-buffered saline, are excellent tissues for screening animals for clenbuterol use. The advantages of Nausch and Galley's[42] findings are that (1) the preparation for immunoassay is essentially a one-step process and (2) retinas (which contain the highest concentrations of clenbuterol) are not used during the screening, and may be saved for confirmatory analyses of animals tested with positive screens.

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Matsumoto *et al.*[68] developed an immunoassay for the determination of clenbuterol in bovine and equine tissues and in bovine milk. The LOD of clenbuterol in milk, muscle, liver, kidney, small intestine, and adipose tissues was 0.1 $\mu g\,kg^{-1}$. Bovine tissue samples fortified with 1 $\mu g\,kg^{-1}$ of clenbuterol had recoveries that varied from 75 to 96%, but recoveries from milk samples were 99%. The authors utilized this method to estimate the clenbuterol withdrawal periods for cattle and horses. Cattle were treated with a bolus dose of either 0.3 or 0.6 $\mu g\,kg^{-1}$ body weight, by intravenous injection, and three animals were slaughtered at days 1, 6, and 9. Tissue clenbuterol levels were detectable only on day 1. Clenbuterol in milk was not detectable after a 2.5-day withdrawal period. Liver contained the highest clenbuterol concentration of the tissues measured, but this group did not measure eye tissues.

Shelver and Smith[32] confirmed that commercial clenbuterol immunoassays cross-react with some, but not all, clenbuterol metabolites. As a result, quantitative clenbuterol immunoassays may differ from determinative methods if substantial concentrations of metabolites are present. For clenbuterol, the parent clenbuterol level is

usually about 40% of the total residue in animals slaughtered with a 0-day withdrawal period.[69–71]

Recently the US FDA approved ractopamine HCl as a feed additive for swine.[72] Although analytical methods exist for quantitative and confirmatory purposes, rapid screening technology is not currently available for widespread use. This has aroused some concern from some regulatory agencies and from portions of the livestock industry. The EU has banned the use of β-agonists and the import of carcasses of animals treated with β-agonists. Some livestock producers may have concern that their products may not be suitable for export, and importers would like to be able to verify that animal carcasses are ractopamine free. In addition, organizers of national, state, and local livestock events have expressed concern that ractopamine could be used in species for which no approval exists.

Several groups have developed either monoclonal[35] or polyclonal antibody-based ractopamine immunoassays.[30,73,74] The cross-reactivity of ractopamine and its metabolite (glucuronides) was measured by Shelver's group[35] (Table 2). All three groups[30,73,74] measured matrix effects in urine, but more work is needed to be able to measure ractopamine residues in edible tissues and carcass components for food safety analyses.

5.2.2 Anti-infectious agents

Anti-infectious agents commonly used for food animals are antibiotics used to treat infections or used as growth promoters, anti-protozoa agents to treat coccidiosis (commonly found in poultry), and anthelmintic agents used to treat nematode infections. For growth purposes, antibiotics are provided continuously in feed or water at low concentrations. Emerging antibiotic resistance has raised concerns about the practice of using 'sub-therapeutic' levels of antibiotics. Allergic reactions are common for some classes of antibiotics, in particular for penicillins, sulfonamides, and cephalosporins, and very low levels of residue in edible products represents a risk to sensitive individuals.[66] Some antibiotics have serious side effects, including hearing loss caused by aminoglycosides and aplastic anemia caused by chloramphenicol, thereby making residual amounts of these antibiotics unacceptable in food. In addition, residue of antibiotics in milk can inhibit the fermentation used to manufacture foods such as cheese and yogurt, resulting in potentially severe economic loss. Concerns about antibiotics in food supplies have led to a number of immunoassays (Table 5) being developed for surveillance purposes.

Benzylpenicilloyl derivatives are formed by the addition of penicillin to the lysine present in proteins and are of interest because of their potential as allergens. Rohner *et al.*[75] developed an immunoassay for benzylpenicilloyl compound groups and penicillin G and used this assay to determine whether benzylpenicilloyl immunoreactive compounds and penicillin G were eliminated in milk at different rates. Because the bacterial inhibition assay used to screen milk samples for penicillin did not detect benzylpenicilloyl residues, the presence of these residues in milk samples could have human health consequences. The assay had an LOD of 1 $\mu g\,kg^{-1}$ for benzylpenicilloyl derivatives and 10 $\mu g\,kg^{-1}$ for penicillin G. A matrix blank was used to generate the calibration curve, but the milk samples were heated at 100 °C for 10 min to eliminate nonspecific reactions. Unheated milk changed the antibody–antigen reaction,

Table 5 Examples of immunoassays developed for anti-infectious agents

Compound [CAS No.]	Matrix	Mab/PAb	Support[a]	LOD ($\mu g\,kg^{-1}$)	Reference
Antibiotics					
Penicillin G [61-33-6]	Milk	PAb	Plate	1	75
	Kidney	PAb	Plate	20	115
Cloxacillin [61-72-3]	Kidney	PAb	Plate	1	115
	Milk	PAb	Plate	10	76
Dicloxacillin [3116-76-5]	Milk	PAb	Plate	30	76
Cephalexin [15686-71-2]	Milk, eggs, tissue	PAb		30, 60, 400	77
Ceftiofur [80370-57-6]	Milk	Mab	Plate	100	78
Chloramphenicol [56-75-7]	Meat, milk			2, 0.5	80
	Meat	PAb	Plate	0.2	115
	Milk	PAb	Dipstick, IF	1	81
Enrofloxacin [93106-60-6]	Milk	PAb		1.6 (1.6-12.5)	116
Erythromycin [114-07-8]	Milk	PAb	Plate	10	82
Spiramycin [8025-81-8]	Milk	PAb	Plate	5.6	117
Gentamicin [1403-66-3]	Milk	Mab/PAb	Nitrocellulose	150	84
	Milk	PAb	Plate	0.7 (LOQ 1.2)	85
	Kidney	PAb	Plate	3.8 (LOQ 7)	
Neomycin [1404-04-2]	Milk	PAb	Plate	3.6 (LOQ 6.3)	85
	Kidney	PAb	Plate	25.4 (LOQ 42)	
Streptomycin [57-92-1]	Milk	PAb	Plate	1.6	118
	Kidney	PAb	Plate	2.5	115
	Milk		IF	2	81
	Milk	PAb	Plate	5.1 (LOQ 9.1)	85
	Kidney	PAb	Plate	27.8 (LOQ 49)	
Dichlorostreptomycin	Milk		IF	5	81
Sulfachlorpyridazine [80-32-0]	Liver, milk, beef, lamb, chicken, turkey, pork, eggs	both	plate	1.2, 0.42, 0.51, 0.18, 1.25, 2.32, 1.0, 3.9	119
Sulfadiazine [68-35-9]	Milk	PAb	Dipstick, IF	12, 30	87
Sulfadimidine [5-68-1]	Milk, tissues (M, K, L)	PAb	Plate	Varied, depend on dilution	89
Sulfamethazine [57-68-1]	Milk			Varied (2–10)	86
	Kidney	PAb	Plate	2	115
	Milk	PAb	Dipstick, IF	10	87
	Milk powder			2, 5	88
Sulfadimethoxine [122-11-2]	Liver	Mab	Plate		91
Sulfamethoxypyridazine [80-35-3]	Milk	PAb	Dipstick, IF	10, 20	87
Sulfathiazole [72-14-0]	Milk	PAb	Test strip, IF	12	90
Tetracycline [60-54-8]	Meat		Plate		92
Trimethoprim [738-70-5]	Milk	PAb	Plate	12.5	117
Anti-parasites					
Lasalocid [11054-70-9]	Chicken muscle/liver	PAb	Plate	0.01/0.09	94
Monensin [17090-79-8]	Liver	PAb	Plate	2.9	97
Salinomycin [53003-10-4]	Liver	Mab	Plate	50 (LOQ)	95
	Chicken muscle/liver	PAb	Plate	0.18/0.17	94
Dimetridazole [551-92-8]	Turkey muscle	Mab	Plate	Low	120
Halofunginone [352464-99-4]	Liver	Mab	Plate	38 (LOQ)	98
Ivermectin [70288-86-7]	Bovine liver	PAb	Plate	1.6	99
Anthelmintics					
Albendazole [54965-21-8]	Calf liver	Mab	Plate	58	103
Fenbendazole [43210-67-9]	Calf liver	Mab	Plate	120	103
Levamisole [14769-73-4]	Meat, milk	PAb	Plate	1	100

[a]IF: immunofiltration.

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reducing the sensitivity of the assay. Results from their study indicated that although benzylpenicilloyl residues remained in cow plasma for extended periods of time, their depletion from milk was simultaneous with penicillin G.

An immunoassay was developed to determine the penicillinase stable isoxazolyl penicillins cloxacillin and dicloxacillin in milk by Usleber *et al.*[76] The assay detected 10 $\mu g\,kg^{-1}$ of cloxacillin and 30 $\mu g\,kg^{-1}$ of dicloxacillin with recoveries of 102% and 84%, respectively. The calibration curve was prepared by fortifying skimmed milk powder (100 $g\,L^{-1}$) with standards. Fortified samples were prepared in pasteurized milk and analyzed directly after decreaming by centrifugation. This immunoassay was performed with minimal sample preparation, probably because the extensive water solubility of the penicillins prevents problems associated with more lipid-soluble analytes.

Kitagawa *et al.*[77] developed a cephalexin immunoassay for use in milk, various hen tissues, and eggs. In addition to cephalexin, the antibody cross-reacted with cephaloglycin and cephalothin. The authors reported the ability to detect 30 $\mu g\,kg^{-1}$ of cephalexin in milk, 60 $\mu g\,kg^{-1}$ in egg yolk, and 400 $\mu g\,kg^{-1}$ in hen tissue. Milk samples exhibited a small matrix effect that could be avoided with a proper blank. Muscle samples were prepared by homogenization in buffer followed by centrifugation. Supernatants were used directly in the immunoassays. The authors used incurred samples prepared by dosing hens with 20 mg of cephalexin per day for 1 week; hens were killed 2 h after the last administration of drug. Eleven tissues were analyzed, with residue levels being 10 times greater in kidney than in the heart. In one of the rare examples of analysis in eggs, these authors extracted the egg albumen with 5% trichloroacetic acid, but no cephalexin was found in these samples. The yolks required a more complex double antibody precipitation prior to analysis and showed significant levels of cephalexin. This work clearly demonstrates the need for tissue-specific isolation procedures and the different distribution of analyte in the animal. The assay was used to follow the time course of the appearance and disappearance of cephalexin in eggs.

A monoclonal antibody-based ELISA has been utilized to determine ceftiofur levels in milk.[78] The authors noted that matrix interference occurred, but a 1 : 100 dilution lowered the interference, and a 1 : 1000 dilution eliminated the matrix interference. Because of the high dilution, samples could not be measured below 1.0 $\mu g\,kg^{-1}$. The assay measured ceftiofur, its major metabolite desfuroylceftiofur, and ceftiofur protein conjugates and has been utilized to measure residues in milk from cows treated with therapeutic doses of the drug. The results from the incurred residue correlated well with a previous study using radiolabeled ceftiofur, confirming the detection of a metabolite that was not detected by HPLC.

Chloramphenicol is a broad-spectrum antibiotic that has been banned from food-animal use in the USA because of evidence that it causes idiosyncratic aplastic anemia in humans.[79] Although this antibiotic has been banned in food animals, its broad activity against pathogenic organisms makes this drug a useful therapeutic tool and valuable in veterinary medicine. The off-label use of chloramphenicol in food animals has been a concern to regulatory organizations worldwide. A commercially available immunoassay for chloramphenicol having an LOD of 2 $\mu g\,kg^{-1}$ in meat and 0.5 $\mu g\,kg^{-1}$ in milk was used to screen a large number of samples ($n = 554$) among 13 laboratories.[80] Meat samples were treated by homogenization and

filtration, with no extraction procedure being necessary. Skim milk was subjected to an SPE cleanup before the immunoassay was conducted. At a cut-off level of 8 $\mu g\,kg^{-1}$, no false-negative values were detected. Positive samples were confirmed by both liquid chromatography (LC) and GC/MS analysis. Märtlbauer *et al.* utilized both a dipstick and an immunofiltration format for the detection of chloramphenicol in milk and reported an LOD of 1 $\mu g\,kg^{-1}$ for both formats.[81] The dipstick format was particularly rapid and convenient for untrained individuals and enabled a quick assessment of milk contamination by chloramphenicol.

Erythromycin belongs to the macrolide group of antibiotics and is used to treat mastitis in cows. Albrecht *et al.*[82] developed an antibody-capture immunoassay for erythromycin in milk samples. Milk samples were skimmed and diluted 1 : 1.5 with phosphate buffer containing Tween 20 prior to analysis. The LOD was 10 $\mu g\,kg^{-1}$ using the mean background + three standard deviations (SD). This group also developed an ELISA to detect spiramycin (another macrolide) in raw milk.[83] The milk was skimmed and treated with ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) prior to the analysis. The LOD was 5.6 $\mu g\,kg^{-1}$, which is well below the EU MRL for spiramycin (150 $\mu g\,kg^{-1}$ in raw milk).

Aminoglycoside antibiotics include amikacin, gentamicin, kanamycin, neomycin, sisomycin and tobramycin. Ara *et al.*[84] generated a dot-ELISA for gentamicin. Dot assays utilize a sandwich format in which a monoclonal antibody acts as a capture antibody and a biotin-labeled polyclonal antibody acts as a detector. Both antibodies were raised against gentamicin–bovine serum albumin. The gentamicin assay was directly applied to raw milk with LOD of 150 $\mu g\,kg^{-1}$. No cross-reactivity was observed with other aminoglycoside antibiotics. Although the sensitivity was not as great as commonly encountered for immunoassays, this format is highly suitable for a field assay.

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Three antibodies generated against gentamicin, neomycin, and streptomycin were reported by Haasnoot *et al.*[85] to detect antibiotic presence in milk and kidney samples. Although their origin was a polyclonal antibody, the assay was generally specific towards targeted compounds. The gentamicin antibody, however, had 25% cross-reactivity toward sisomycin, and the streptomycin antibody had 150% cross-reactivity to the chemically closely related dihydrostreptomycin. As described below, antibodies directed against streptomycin commonly cross-react strongly with dihydrostreptomycin because of their structural similarity. This is not a serious problem since both are active antibacterials with similar biochemical properties. Milk samples were decreamed and diluted 10-fold in buffer, resulting in an LOQ of the mean background + 6 SD. This limit corresponded to 1.2, 6.3, and 9.1 $\mu g\,kg^{-1}$ for the gentamicin, neomycin, and streptomycin ELISAs, respectively. Fortified milk samples had variable recoveries and high relative SDs, so the authors suggested that the assay be used as a semi-quantitative screening tool. The sensitivity of the assay was well below the MRL for each analyte. When the assay was used to screen incurred samples, some animals that had aminoglycoside levels exceeding the established MRL in kidney were detected. The authors suggested that because of the long retention time of these drugs in kidney and the unpredictable variation of residues in diseased animals, the organs of sick animals should be condemned at inspection and excluded from routine screening. This is an excellent example of the interaction of pharmacokinetics and analytical chemistry, where knowledge in both fields can be used to a great advantage in the development and application of meaningful analysis.

Many groups have developed immunoassays for sulfonamides. These compounds were a problem in the late 1980s when they were commonly found in the milk supplies in the USA. Subsequently, a great deal of effort has been required to develop rapid, reliable assays for the large number of chemical variations of sulfonamides.

Medina *et al.*[86] evaluated a number of commercially available immunoassay kits for the detection of sulfamethazine. Several of the kits were qualitative and had a visual limit of detection from 2 to 10 $\mu g\,kg^{-1}$. Other kits were quantitative and detection limits were usually below 1 $mg\,kg^{-1}$. Incurred samples were obtained from cows dosed orally with 1.5 $mg\,kg^{-1}$ body weight of sulfamethazine. Immunoassay results were verified by high-performance thin-layer chromatography and HPLC with electrochemical detection. Kits were evaluated for the sample preparation effects, matrix interferences, and calibration and detection range. Although there was qualitative agreement among the kits, there were also rather large quantitative differences between kits and even between the two confirmation methods. Sample preparation for the immunoassays was rather simple (warming and centrifugation), whereas the instrumental methods required a three-column SPE cleanup. Some of the quantitative differences between the instrumental methods and the immunoassays were ascribed to the known sensitivity of the immunoassays to sulfamethazine metabolites.

Test strip and immunofiltration devices were developed by Ostermaier *et al.*[87] to detect sulfadiazine, sulfamethazine, and sulfamethoxypyridazine in milk. Direct competitive immunoassay was utilized with sulfonamide–horseradish peroxidase as the detector. The LOD for sulfamethazine for both the dipstick and immunofiltration was 10 $\mu g\,kg^{-1}$; for sulfadiazine, the LOD was 12 $\mu g\,kg^{-1}$ for the dipstick and 30 $\mu g\,kg^{-1}$ for immunofiltration. For sulfamethoxypyridazine, the LOD was 10 $\mu g\,kg^{-1}$ for the dipstick and 20 $\mu g\,kg^{-1}$ for immunofiltration. The devices were found to be suitable for on-site use with undiluted milk.

Lopez-Avila and Benedicto[88] combined SFE with ELISA to determine sulfamethazine in powdered milk. Various conditions were tested in order to achieve quantitative extraction of sulfamethazine. Variations in extraction pressure, temperature, extraction period, and the presence of organic modifier resulted in extraction efficiencies of 0–92%. Once optimal extraction conditions had been developed, a commercially available ELISA was utilized to determine sulfamethazine concentrations. The LOD was 2.5 $\mu g\,kg^{-1}$, and satisfactory recoveries were obtained at levels from 5 to 15 $\mu g\,kg^{-1}$.

Franek *et al.*[89] developed an immunoassay for sulfadimidine, but they found that milk samples required dilution (1 : 100), and tissues required dilutions of 1 : 200 to 1 : 4000. The LOD was 0.02 $\mu g\,kg^{-1}$ from the buffer calibration curve with the IC_{50} at 0.15 $\mu g\,kg^{-1}$. The assay measured levels in milk from 10 to 100 $\mu g\,kg^{-1}$ with satisfactory precision. In swine muscle, kidney, and liver samples, levels from 20 to 500 $\mu g\,kg^{-1}$ could be measured when 2 g of tissue were homogenized with 20 mL of buffer and then diluted 1 : 20.

A sulfathiazole immunoassay[90] was utilized to determine residues present in raw milk. The LOD was found to be 12 $\mu g\,kg^{-1}$ (based on 80% B_0); however, comparison of the calibration curve from an aqueous solution with a raw milk calibration curve indicated a significant matrix effect.

Muldoon *et al.*[91] developed a monoclonal-based competitive inhibition enzyme-linked immunosorbent assay (cELISA) for sulfadimethoxine. The group compared

cELISA and HPLC results from extracts obtained from incurred chicken livers using various extraction solvents. When liver extracts were diluted 1 : 25, the calibration curve could be superimposed on the calibration curve created in buffer. When the extraction techniques for HPLC and cELISA used the same organic solvent (acetonitrile–water or acetone), the two methods were highly correlated ($r^2 = 0.976$ and 0.912, respectively). When cELISA and HPLC analyses were performed after simple aqueous extraction, the correlation between the two methods was poor ($r^2 = 0.609$). The correlation between the two methods was improved by ultrafiltration of the aqueous extract to eliminate sulfadimethoxine–protein conjugates ($r^2 = 0.909$). Different extraction techniques will influence the agreement between specific instrumental methods and ELISAs owing to the co-extraction of metabolites or protein conjugates that may react with the antibody but are not measured by the instrumental method. In this case, the confirmatory method (HPLC) was not able to measure the sulfadimethoxine–protein conjugate. Therefore, if total residue (parent + metabolite) is needed, cELISA with aqueous extraction produces a more accurate result than HPLC.

A commercial immunoassay for compounds within the tetracycline family has been compared with a microbiological inhibition assay method for tetracycline residues in pork and chicken muscle tissues. Samples determined to be positive for tetracycline by a microbiological screen were used to determine the correlation (not defined in the original paper) between the two methods.[92] Of 21 microbiologically positive chicken samples, 19 were positive with the ELISA ($B/B_0 < 0.75$); 18 samples were found to contain doxycycline after confirmatory LC/fluorescence or LC/MS/MS analyses were performed. The author found that the immunoassay results were poorly correlated with the HPLC method ($r^2 = 0.74$), although the inhibition zone of the microbial assay size correlated reasonably well ($r^2 = 0.94$) with the HPLC method. In pork, both the HPLC method and the ELISA gave a poor correlation with the inhibition zone size ($r^2 = 0.72$). The ELISA kit could detect all four commonly used tetracyclines (oxytetracycline, chlortetracycline, tetracycline, and doxycycline), although the kit was least sensitive for doxycyline, which could account for the relatively low correlation between the ELISA and the confirmatory methods. The authors speculated that the increased levels of fat in the pork samples might have reduced diffusion into the media, thereby lowering correlation with the confirmatory method.

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5.2.3 Anti-protozoa agents

Anti-protozoa agents are utilized to treat diseases such as coccidiosis, which affects many farm animals, particularly poultry. Coccidiostats include polyether monocarboxylic acid ionophores and other types of compounds. Polyether monocarboxylic acid ionophores include monesin, narasin, lasalocid, and salinomycin. The most common of these is salinomycin. Nonionophore coccidiostats include dimetridazole and halofunginone. Stanker *et al.*[93] reviewed immunoassays available for coccidiostatic agents.

Kennedy *et al.*[94] developed an immunoassay for salinomycin and utilized the IA to check the depletion of salinomycin in chicken muscle and liver. The limit of quantitation was 0.31 $\mu g\,kg^{-1}$ for muscle and 0.29 $\mu g\,kg^{-1}$ for liver; these levels were based on the assay response of negative samples + 6 SD. The antibody cross-reacted with narasin but did not recognize lasalocid, maduramicin, and monensin.

Cross-reactivity with narasin was not surprising, because narasin or salinomycin differ by the presence of a methyl group on narasin that is absent on salinomycin.

Muldoon *et al.*[95] developed a monoclonal antibody based immunoassay for salinomycin to determine its residue in chicken liver. The liver samples were extracted with methanol, and the methanol layer was extracted with methylene chloride. The liver extract was then diluted 1 : 100 in order to minimize the matrix effects. Since a significant matrix effect remained, a B/B_0 transformation was used for quantitation. This transformation minimized some matrix effects but reduced the linear range or sensitivity of the method. Both fortified and incurred liver samples were analyzed with a confirmatory HPLC method. The fortified liver samples showed very similar curves for both HPLC and ELISA, although the ELISA curve showed better linearity at low levels. The LOQ for both assays was 50 $\mu g\,kg^{-1}$ in liver tissue, but the HPLC assay deviated from linearity below 100 $\mu g\,kg^{-1}$. An incurred residue study was carried out using a total of 45 chickens (15 in a control dose, 15 receiving 66 $mg\,kg^{-1}$, and 15 animals receiving 132 $mg\,kg^{-1}$; five of each dose were killed at 0, 18, and 72 h). The results of the ELISA were highly correlated with HPLC, although the ELISA results were generally higher. Using HPLC as the standard, the ELISA produced one false negative and two false positives, all near the limit of detection of the HPLC method.

Kennedy *et al.*[96] developed a lasalocid immunoassay for application to residues in chicken meat and liver samples. The antibody was specific and did not cross-react with salinomycin, maduramicin, or monensin. Sample preparation consisted of homogenization in aqueous acetonitrile, removal of fat from an aliquot of the aqueous acetonitrile by hexane extraction, and evaporation of acetonitrile. The sample was then reconstituted with assay buffer. Liver required an additional solid phase extraction step. The LOQ was 0.02 $\mu g\,kg^{-1}$ for muscle and 0.15 $\mu g\,kg^{-1}$ for liver. These workers were able to use the system to determine the half-life of lasalocid in the tissues.

Crooks *et al.*[97] developed a monensin immunoassay for the detection of residues in broiler livers. Livers were homogenized in aqueous acetonitrile, subsequently extracted with sodium hydroxide followed with hexane–diethyl ether, and the remaining solvent was evaporated before reconstitution in buffer for analysis. The LOD (mean + 3 SD) was 2.9 $\mu g\,kg^{-1}$, and the LOQ (mean + 6 SD) was 4.6 $\mu g\,kg^{-1}$. Incurred samples showed considerable animal-to-animal variation, but all samples were below the detection limit after 3 days.

Beier *et al.*[98] compared halofuginone residue measurements by HPLC and cELISA using fortified and incurred liver samples. They found that cELISA and HPLC determinations gave good agreement ($r^2 = 0.98$ for fortified samples and 0.94 for incurred samples), despite using a simple cleanup procedure for cELISA relative to an elaborate extraction method for HPLC samples. The cELISA did give occasional inconsistent recoveries that might be expected given the very simple sample preparation. The LOQ (based on 80% B/B_0) for cELISA was 38 $\mu g\,kg^{-1}$. The cELISA assay is suitable for the determination of acceptable levels for halofuginone, which has an MRL of 160 $\mu g\,kg^{-1}$.

Ivermectin, a macrocyclic lactone, is also utilized to control parasites. An immunoassay was developed to determine ivermectin residues in bovine liver by Crooks *et al.*[99] The sample preparation procedure was complex, involving tissue homogenization in acetonitrile, centrifugation, extraction with hexane (to remove lipids), evaporation and reconstitution in ethyl acetate, and passage through an SPE column followed

by evaporation of solvent, reconstitution in buffer. The authors attributed the need for this procedure to the lipophilicity of ivemectin. The LOD was 1.6 $\mu g\,kg^{-1}$ using a background mean + 3 SD, and the LOQ was 2.9 $\mu g\,kg^{-1}$ using a background mean + 6 SD. The antibody cross-reacted with doramectin (13.5%) but did not recognize milbemycin or moxidectin. The intra- and inter-immunoassay variation determined from spiked samples was below 10% and 15%, respectively. Four steers were treated with a pour-on ivermectin application of 0.5 mg kg^{-1} body weight and were killed after 7, 14, 21 and 28 days. Liver samples measured by immunoassay and HPLC correlated well, with $r^2 = 0.98$. The ivermectin concentration in liver sample residues decreased rapidly beginning 7 days after treatment at 52 $\mu g\,kg^{-1}$ and decreasing to 1–4 $\mu g\,kg^{-1}$.

5.2.4 Anthelmintics

Anthelmintic agents have been utilized to treat a multitude of nematode infections. These include roundworms, tapeworms, and lungworms in cattle and swine. Two classes of compounds included as anthelmintic agents will be discussed here, levamisole and thiabendazoles (thiabendazoles can also act as pesticides). Thiabendazoles can cause nephrotoxicity, teratogenesis, and immunosuppression and can disrupt endocrine balance. Because of these toxicities, residues of these compounds in food animals are of food safety concern.

Silverlight and Jackman[100] developed an immunoassay for levamisole in meat and milk. The LOD in both milk and meat samples was 1 $\mu g\,kg^{-1}$. The assay was applied to milk directly, and muscle samples required only homogenization in the presence of 10-fold of buffer prior to analysis. The linear range of the assay was between 5 and 50 $\mu g\,kg^{-1}$ for meat and between 0.2 and 25 $\mu g\,kg^{-1}$ for milk. The linear range of the assay was below the MRL for milk (10 $\mu g\,kg^{-1}$) and meat (50 $\mu g\,kg^{-1}$).

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Benimidazoles are also used as anthelmintics and(or) fungicides and could be found in meats if the animal is slaughtered too soon after administration. Using a simple extraction procedure (water followed by centrifugation), rapid screening assays have been developed to determine benzimidazole residues in bovine liver.[101] Brandon *et al.* used a commercial thiabendazole immunoassay kit developed in their laboratory[102] which recognized thiabendazole, its 5-hydroxy metabolite, and cambendazole. The thiabendazole kit was able to distinguish correctly both incurred and fortified samples from blanks but did not recognize the methylbenzimidazole carbamates (MBCs). A second monoclonal antibody that recognized albendazole, fenbendazole, and oxfendazole (major anthelmintic drugs) was also used in the study.[103] In order to optimize the assay, the extraction was extended to 1 h and the sample was centrifuged at 20 000 *g* (10-min extraction and 4500 *g* were not sufficient). The monoclonal antibody for MBCs recognized parent compounds and the oxidized metabolites, both in fortified samples and incurred samples, correctly identifying all positive samples with relative standard deviations less than 10%.

5.3 Other therapeutic agents

Furosemide is a diuretic agent used to treat edema in both human and veterinary medicine and requires a 48-h withdrawal period for milk produced by cattle

treated with this drug. Stanker *et al.*[104] developed a monoclonal antibody toward this compound and applied a competitive inhibition immunoassay either to cow's milk that was fortified with furosemide or milk from incurred samples. The immunoassay produced accurate results when the levels were below 100 $\mu g\,kg^{-1}$. Above 100 $\mu g\,kg^{-1}$ the immunoassay overestimated the furosemide compared with the HPLC method. The only treatment for milk samples for ELISA testing was dilution, whereas for the HPLC sample, a simple extraction was used. The HPLC method was much less sensitive than the ELISA, which gave an LOD of 0.5 $\mu g\,kg^{-1}$ and an LOQ of 2 $\mu g\,kg^{-1}$.

6 Other antibody-based technologies

Immunoaffinity chromatography (IAC) coupled with GC or LC can be utilized for either single- or multi-residue analysis. Traditionally, sample preparation for GC or LC is labor intensive. Immunoaffinity columns serve to specifically retain analytes, while interferences are rinsed off the column. Analytes are specifically eluted from immunoaffinity columns and are then assayed using instrumental methods. This approach also works for multi-residue analysis when several antibodies targeting related analytes are used in the columns. IAC may save time and reduce solvent consumption, yet still be highly specific. In addition, the generation of large quantities of homogeneous monoclonal antibodies allows for the development of highly consistent single- or multiple-use columns.

Van Ginkel *et al.*[105] utilized multi-IAC for the cleanup of multi-anabolic residues from meat. They found that with the proper defatting of samples prior to IAC, individual columns could be used more than 25 times. On-line IAC was coupled with reversed-phase LC to isolate four fluoroquinolones (ciprofloxacin, difloxacin, enrofloxacin, and sarafloxacin) simultaneously from milk and chicken liver.[106] IAC with two different antibodies was used to isolate multiple sulfa drugs from milk.[107] Antibodies against sulfadiazine and sulfamethazine gave satisfactory binding for sulfadiazine, sulfathiazole, sulfamerazine, and sulfamethazine, clearly demonstrating that modest cross-reactivity may allow the isolation of multiple analytes.[108] Again, with multi-residue analysis, success depends upon the suitable design of the hapten used to generate the antibody. IAC was also used by Li *et al.* to clean up sulfonamides (sulfamonomethoxine, sulfadimethoxine, and sulfaquinoxaline) from swine meat.[109] These workers prepared antibodies from sulfanilamide with a spacer containing an aromatic ring that created an antibody capable of binding a number of different sulfonamides rather than a single compound. Samples were analyzed after simple homogenization in aqueous methanol, subsequent IAC, followed by HPLC. Sulfamonomethoxine, sulfadimethoxine, and sulfaquinoxaline were successfully quantified at concentrations of 10–100 $\mu g\,kg^{-1}$ for each of the drugs. A commercially available clenbuterol IAC cartridge was utilized by Lawrence *et al.*[110] for beef liver and muscle tissue cleanup. The column is rugged and can be re-used up to 10 times without significant loss of binding capacity. These workers used an ultrasonic extraction procedure, weak cation SPE, and an IAC cartridge and analyzed the sample with HPLC. They could quantitate at 2 $\mu g\,kg^{-1}$ and estimated the LOD to be 0.3 $\mu g\,kg^{-1}$.

7 Conclusion

The use of immunoassays for the determination of pesticides and veterinary medicines in food animals has increased since the early 1990s. The advantages of simple analysis, quick results, and high throughput make immunoassays a powerful technique for problematic matrices commonly encountered in animal agriculture. Careful development and validation are required to obtain accurate results, however. This review has demonstrated that most immunochemical techniques have been designed for use with milk samples, but a number of applications have also been developed for liver and muscle samples. The development of immunoassay techniques for residue analysis in eggs has clearly not been pursued to the extent of other edible tissues.

Immunoassays are most often utilized as screening techniques for food-animal applications, with positive samples subsequently being analyzed with instrumental confirmatory methods. Correlations between the immunoassay and instrumental method are often determined using spiked samples, but incurred samples provide a more rigorous test, particularly if significant amounts of metabolites are present. SFE utilized for pre-immunoassay treatment shows some promise but probably has not reached its full potential. Emerging new techniques such as molecular imprint polymers, immunoaffinity chromatography, and biosensors are interesting approaches that could be significant players in future immunoassay development. The literature has established the importance of immunoassays in the difficult and challenging field of residue analysis in food products.

8 Abbreviations

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ELISA	enzyme-linked immunosorbent assay
EU	European Union
FDA	Food and Drug Adminstration
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
HPLC	high-performance liquid chromatography
IA	immunoassay
IAC	immunoaffinity chromatography
LC	liquid chromatography
LC/MS	liquid chromatography/mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
LSC	liquid scintillation counting
MRL	maximum residue limit
SFE	supercritical fluid extraction
SPE	solid-phase extraction.

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approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

References

1. J.R. Crowther 'Methods in Molecular Biology. Vol. 149: The ELISA Guidebook,' Humana Press, Totowa, NJ (2000).
2. S.S. Deshpande (ed.), 'Enzyme Immunoassays: from Concept to Product,' Chapman & Hall, New York (1996).
3. E.P. Diamandis and T.K. Christopoulos (eds), 'Immunoassay' Academic Press, San Diego (1996).
4. D.S. Aga and E.M. Thurman (eds), 'Immunochemical Technology for Environmental Applications,' ACS Symposium Series 657, American Chemical Society, Washington, DC (1997).
5. R.C. Beier and L.H. Stanker (eds), 'Immunoassays for Residue Analysis,' ACS Symposium Series 621, American Chemical Society, Washington, DC (1995).
6. J.A. Gabaldon, A. Maquieira, and R. Puchades, *Crit. Rev. Food Sci. Nutr.,* **39**, 519 (1999).
7. J.M. Mitchell, M.W. Griffiths, S.A. McEwen, W.B. McNab, and A.J. Yee, *J. Food Protect.,* **61**, 742 (1998).
8. L.H. Stanker, 'Residue Detection by Immunoassay,' in "Analysis of Contaminants in Edible Aquatic Resources," ed. J.W. Kiceniuk and S. Ray, VCH, New York, pp. 523–537 (1994).
9. S.S. Gazzaz, B.A. Rasco, and F.M. Dong, *Crit. Rev. Food Sci. Nutr.,* **32**, 197 (1992).
10. U. Samarajeewa, C.I. Wei, T.S. Huang, and M.R. Marshall, *Crit. Rev. Food Sci. Nutr.,* **29**, 403 (1991).
11. H.A. Kuiper, M.Y. Noordham, M.M.H. van Dooren-Flipsen, R. Schilt, and A.H. Roos, *J. Anim. Sci.,* **76**, 195 (1998).
12. F. Ramos, M. Conceicao Castilho, and M.I. Noronnha da Silveira, *J. AOAC Int.,* **81**, 544 (1998).
13. C.T. Elliott, J.D.G. McEvoy, W.J. McCaughey, D.H. Shortt, and S.R.H. Crooks, *Analyst,* **118**, 447 (1993).
14. D.M. Henricks and A.K. Torrence, *J. Anim. Sci.,* **46**, 654 (1977).
15. B. Hoffmann and H. Karg, *Environ. Qual. Saf. Suppl.,* **5**, 181 (1976).
16. D.M. Henricks, *J. Toxicol. Environ. Health,* **1**, 617 (1976).
17. P. Singh, B.P. Ram, and N. Sharkov, *J. Agric. Food Chem.,* **37**, 109 (1989).
18. D.E. Dixon-Holland and S.E. Katz, *J. AOAC Int.,* **71**, 1137 (1988).
19. D.E. Dixon-Holland and S.E. Katz, *J. AOAC Int.,* **72**, 447 (1989).
20. W. Haasnoot, L. Streppel, G. Cazemier, M. Salden, P. Stouten, M. Essers, and P. van Wichen, *Analyst,* **121**, 1111 (1996).
21. K.E.I. Vanoosthuyze, C.J.M. Arts, and C.H. Van Peteghem, *J. Agric. Food Chem.,* **45**, 3129 (1997).
22. W. Haasnoot, A.R.M. Hamers, G.D. van Bruchem, R. Schilt, and L.M.H. Frijns, *Food Saf. Qual. Assur. Appl. Immuno. Syst.,* **4**, 237 (1992).
23. R.I. McConnell, A. McCormick, J.V. Lamont, and S.P. Fitzgerald, *Food Agric. Immunol.,* **6**, 147 (1994).
24. C.T. Elliott, S.R.H. Crooks, and W.J. McGaughey, *Vet. Rec.,* **137**, 643 (1995)
25. P. Delahaut, M. Dubois, I. Pri-bar, O. Buchman, G. Degand, and F. Ectors, *Food Addit. Contam.,* **8**, 43 (1991).
26. G. Degand, A. Bernes-Cuyckaerts, and G. Maghuin-Rogister, *J. Agric. Food Chem.,* **40**, 70 (1992).
27. E. Petruzzelli, A. Ius, S. Berta, M. Dovis, and A. Albertini, *Food Agric. Immunol.,* **8**, 3 (1996).
28. J.H. Skerritt and B.E. Amita Rani, 'Detection and removal of sample matrix effects in agrochemical immunoassays,' in "Immunoassay for Residue Analysis," ed. R.C. Beier and L.H. Stanker, American Chemical Society, Washington, DC, Chapter 3, pp. 29–43 (1996).
29. K.W. Hinchcliff, A.T. Jernigan, D.W. Upson, and P.D. Constable, *Vet. Clin. N. Am. Food Anim. Pract.,* **7**, 633 (1991).

30. W. Haasnoot, P. Stouten, A. Lommen, G. Cazemier, D. Hooijerink, R. Schilt, *Analyst,* **119**, 2675 (1994).
31. I. Yamamoto and K. Iwata, *J. Immunoassay,* **3**, 155 (1982).
32. W.L Shelver and D.J. Smith, *Food Addit. Contam.,* **17**, 837 (2000).
33. D. Zalko, G. Bories, and J. Tulliez, *J. Agric. Food Chem.,* **46**, 1935 (1998).
34. J.E. Dalidowicz, T.D. Thomson, and G.E. Babbitt, 'Ractopamine hydrochloride, a phenethanolamine repartitioning agent,' in "Xenobiotics in Food Producing Animals Metabolism and Residues," ed. D.H. Hutson, D.R. Hawkins, G.D. Paulson, and C.B. Struble, American Chemical Society, Washington, DC, Chapter 16, pp. 234–243 (1992).
35. W.L. Shelver, D.J. Smith, and E.S. Berry, *J. Agric. Food Chem.,* **48**, 4020 (2000).
36. C.T. Elliott, H.D. Shortt, D.G. Kennedy, and W.J. McCaughey, *Vet. Q.,* **18**, 41 (1996).
37. S. Hahnau and B. Jülicher, *Food Addit. Contam.,* **13**, 259 (1996).
38. H.H.D. Meyer, *Ann. Rech. Vet.,* **22**, 299 (1991).
39. S. Collins, M. O'Keeffe, and M.R. Smyth, *Analysis,* **119**, 2671 (1994).
40. A.L. Wicker, M.P. Turberg, and M.R. Coleman, *Analyst,* **120**, 2879 (1995).
41. W.N. Sawaya, K. Lone, A. Husain, B. Dashti, and T. Saeed, *Food Control,* **11**, 1 (2000).
42. I. Nausch, and K. Galley, *Arch. Lebensmittelhyg.,* **50**, 104 (1999).
43. H.H.D. Meyer and L.M. Rinke, *J. Anim. Sci.,* **69**, 4538 (1991).
44. N.E. Weber, 'Use of xenobiotics in food-producing animals in the United States, regulatory aspects,' in "Xenobiotics and Food Producing Animals," ed. D.H. Hutson, D.R. Hawkins, G.D. Paulson, and C.B. Struble, American Chemical Society, Washington, DC, Chapter 2, pp. 17–25 (1992).
45. M.M.L. Aerts, A.C. Hogenboom, and U.A.Th. Brinkman, *J. Chromatogr. B,* **667**, 1 (1995).
46. C.A. Kan and M. Petz, *J. Agric. Food Chem.,* **48**, 6397 (2000).
47. D. Arnold and A. Somogyi, *J. Assoc. Off. Anal. Chem.,* **68**, 984 (1985).
48. G.D. Paulson, V.J. Feil, J.M. Giddings, and C.H. Lamourex, *Xenobiotica,* **22**, 925 (1992).
49. J.M Van Emon and C.L. Gerlach, *J. Microbiol. Methods,* **32**, 121 (1998).
50. S.J. Lehotay and R.W. Miller, *J. Environ. Sci. Health B,* **29**, 395 (1994).
51. J.E. France and J.W. King, *J. Assoc. Off. Anal. Chem.,* **74**, 1013 (1991).
52. J.W. King and K.S. Nam, 'Coupling enzyme immunoassay with supercritical fluid extraction,' in "Immunoassays for Residue Analysis," ed. R.C. Beier and L.H. Stanker, American Chemical Society, Washington, DC, Chapter 34, pp. 422–438 (1996).
53. S.J. Lehotay and R.J. Argauer, *J. Agric. Food Chem.,* **41**, 2006 (1993).
54. L.H. Stanker, B. Watkins, M. Vanderlaan, R. Ellis, and J. Rajan, 'Analysis of heptachlor and related cyclodiene insecticides on food products,' in "Immunoassays for Trace Chemical Analysis," ed. M. Vanderlaan, L.H. Stanker, B.S. Watkins, and D.W. Roberts, American Chemical Society, Washington, DC, Chapter 12, pp. 108–123 (1991).
55. L.H. Stanker, C. Bigbee, J. Van Emon, B. Watkins, R.H. Jensen, C. Morris, and M. Vanderlaan, *J. Agric. Food Chem.,* **37**, 834 (1989).
56. R.J. Bushway, B. Perkins, S.A. Savage, S.L. Lekousi, and B.S. Ferguson, *Bull. Environ. Contam. Toxicol.,* **42**, 899 (1989).
57. R.J. Bushway, L.B. Perkins, H.L. Hurst, and B.S. Ferguson, *Food Chem.,* **43**, 283 (1992).
58. M. Franek, V. Kolar, and S.A. Eremin, *Anal. Chim. Acta,* **311**, 349 (1995).
59. M. Schwalbe, E. Dorn, and K. Beyerman, *J. Agric. Food Chem.,* **32**, 734 (1984).
60. J. Van Emon, J. Seiber, and B. Hammock, *Bull. Environ. Contam. Toxicol.,* **39**, 490 (1987).
61. B.S. Rutherford, R.C. Gardner, S.D. West, C.K. Robb, and S.C. Dolder, *J. Agric. Food Chem.,* **48**, 4428 (2000).
62. H.L. Beasley, T. Phongkham, M.H. Daunt, S.L. Guihot, and J.H. Skerritt, *J. Agric. Food Chem.,* **46**, 3339 (1998).
63. D.E. Moody, D.L. Hancock, and D.B. Anderson, 'Phenethanolamine repartitioning agents,' in "Farm Animal Metabolism and Nutrition," ed. J.P.F. D'Mello, CAB International, Wallingford, Chapter 4, pp. 65–96 (2000).
64. D.B. Anderson, E.L. Veenhuizen, D.J. Jones, A.L. Schroeder, and D.L. Hancock, 'The use of phenethanolamines to reduce fat and increase carcass leanness in meat animals,' in "Fat and

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Cholesterol Reduced Foods: Technologies and Strategies," ed. G. Bray, Portfolio Publishing, Wood, TX, Chapter 5, pp. 43–73 (1990).
65. G.A. Mitchell and G. Dunnavan, *J. Anim. Sci.,* **76**, 208 (1998).
66. S.F. Sundlof and J. Cooper, 'Human health risks associated with drug residues in animal-derived foods,' in "Veterinary Drug Residues, Food Safety," ed. W.A. Moats and M.B. Medina, American Chemical Society, Washington, DC, pp. 5–17 (1996).
67. C.T. Elliott, W.J. McCaughey, S.R.H. Crooks, J.D.G. McEvoy, and D.G. Kennedy *Vet. Q.,* **17**, 100 (1995).
68. M. Matsumoto, N. Maruyama, H. Watanabe, T. Kiruchimara, Y. Kida, and A. Tsuji, *J. Food Hyg. Soc. Jpn.,* **41**, 48 (2000).
69. D.J. Smith and G.D. Paulson, *J. Anim. Sci.,* **75**, 454 (1997).
70. D.J. Smith, *J. Anim. Sci.,* **76**, 173 (1998).
71. D.J. Smith, *J. Anim. Sci.,* **78**, 2903 (2000).
72. FDA. *Fed. Regist.,* **65**, 4111 (2000).
73. W.L. Shelver and D.J. Smith, *J. Immunoassay,* **21**, 1 (2000).
74. C.T. Elliott, C.S. Thompson, C.J.M. Arts, S.R.H. Crooks, M.J. van Baak, E.R. Verheij, and G.A. Baxter, *Analyst,* **123**, 1103 (1998).
75. P. Rohner, M. Schallibaum, and L. Nicolet, *J. Food Protect.,* **48**, 59 (1985).
76. E. Usleber, M. Lorber, M. Straka, G. Terplan, and E. Martlbauer, *Analyst,* **119**, 2765 (1994).
77. T. Kitagawa, Y. Gotoh, K. Uchiyama, Y. Kohri, T. Kinoue, K. Fujiwara, and W. Ohtani, *J. Assoc. Off. Anal. Chem.,* **71**, 915 (1988).
78. L.H. Stanker, S. Buckley, M. Muldoon, W.A. Moats, and C. Braswell, *Food Agric. Immunol.,* **10**, 121 (1998).
79. J. A. Settepani, *J. Am. Vet. Med. Assoc.,* **184**, 930 (1984).
80. H.J. Keukens, H.M.L. Alerts, W.A. Traag, J.F.M. Nouws, W.G. Deruig, W.M.J. Beek, and J.M.P. Den Hartog, *J. Assoc. Off. Anal. Chem.,* **75**, 245 (1992).
81. E. Martlbauer, E. Usleber, E. Schneider, R. Dietrich, *Analyst,* **119**, 2543 (1994).
82. U. Albrecht, H.G. Walte, and P. Hammel, *Kieler Milchwirtsch. Forschungsber.,* **50**, 163 (1998).
83. U. Albrecht, P. Hammer, and W. Heescen, *Milchwissenschaft,* **51**, 515 (1996).
84. J. Ara, Z. Gans, R. Sweeney, and B. Wolf, *J. Clin. Lab. Anal.,* **9**, 320 (1995).
85. W. Haasnoot, P. Stouten, G. Cazemier, A. Lommen, J.F.M. Nouws, and H.J. Keukens, *Analyst,* **124**, 301 (1999).
86. M.B. Medina, R.A. Barford, M.A. Palumbo, and L.D. Rowe, *J. Food Protect.,* **55**, 284 (1992).
87. S. Ostermaier, E. Schneider, E. Usleber, E. Martlbauer, and G. Terplan, *Food Agric. Immunol.,* **7**, 253 (1995).
88. V. Lopez-Avila and J. Benedicto, 'Determination of veterinary drugs in dry milk powder by supercritical fluid extraction–enzyme linked immunosorbent assay,' in "Veterinary Drug Residues Food Safety," ed. W.A. Moats and M.B. Medina, American Chemical Society, Washington, DC, Chapter 15, pp. 145–148 (1995).
89. M. Franek, V. Kolar, A. Deng, and S. Crooks, *Food Agric. Immunol.,* **11**, 339 (1999).
90. C.A. Thomson, and P. Sporns, *J. Food Sci.,* **60**, 409 (1995).
91. M.T. Muldoon, S.A. Buckley, S.S. Deshpande, C.K. Holtzapple, R.C. Beier, and L.H. Stanker, *J. Agric. Food Chem.,* **48**, 545 (2000).
92. K. De Wasch, L. Okerman, S. Crouble, H.D. Brabander, J.V. Hoof, and P. De Backer, *Analyst,* **123**, 2737 (1998).
93. L.H. Stanker, M.H. Elissalde, L.D. Rowe, R.C. Beier, M.I.A. El-Aziz Nasr, *Food Agric. Immunol.,* **6**, 45 (1994).
94. D.G. Kennedy, W.J. Blanchflower, and B.C. O'Dornan, *Food Addit. Contam.,* **12**, 93 (1995).
95. M.T. Muldoon, M.H. Elissalde, R.C. Beier, and L.H. Stanker, *J. Agric. Food Chem.,* **43**, 1745 (1995).
96. D.G. Kennedy, W.J. Blanchflower, and B.C. O'Dornan, *Food Addit. Contam.,* **12**, 83 (1995).
97. S.R.H. Crooks, I.M. Traynor, C.T. Elliott, and W.J. McCaughey, *Analyst,* **122**, 161 (1997).
98. R.C. Beier, T.J. Dutko, S.A. Buckley, M.T. Muldoon, C.K. Holtzapple, and L.H. Stanker, *J. Agric. Food Chem.,* **46**, 1049 (1998).
99. S.R.H. Crooks, A.G. Baxter, I.M. Traynor, C.T. Elliott and W.J. McCaughey, *Analyst,* **123**, 355 (1998).

100. J.J. Silverlight, and R. Jackman, *Analyst,* **119**, 2705 (1994).
101. D.L. Brandon, K.P. Holland, J.S. Dreas, and A.C. Henry, *J. Agric. Food Chem.,* **46**, 3653 (1998).
102. D.L. Brandon, R.G. Binder, R.E. Wilson, and W.C. Montague Jr, *J. Agric. Food Chem.,* **40**, 1722 (1992).
103. D.L. Brandon, R.G. Binder, A.H. Bates, and W.C. Montague Jr, *J. Agric. Food Chem.,* **42**, 1588 (1994).
104. L.H. Stanker, M.T. Muldoon, S.A. Buckley, C. Braswell, C. Kamps-Holtzappe, R.C. Beier, *J. Agric. Food Chem.,* **44**, 2455 (1996).
105. L.A. Van Ginkel, R.W. Stephany, H.J. Van Rossum, H.M. Steinbuch, G. Zomer, E. Van Deheeft, and P.J.M. De Jong, *J. Chromatogr. B,* **489**, 111 (1989).
106. C.K. Holtzapple, S.A. Buckley, and L.H. Stanker, *J. Assoc. Off. Anal. Chem.,* **82**, 607 (1999).
107. C.K. Holtzapple, S.A. Buckley, and L.H. Stanker, *J. Agric. Food Chem.,* **47**, 2963 (1999).
108. E. Martlbauer, R. Dietrich, and E. Usleber, 'Immunoaffinity chromatography as a tool for the analysis of antibiotics and sulfonamides,' in "Veterinary Drug Residues, Food Safety," ed. W.A. Moats and M.B. Medina, American Chemical Society, Washington, DC, Chapter 13, pp. 121–131 (1995).
109. J.S. Li, X.W. Li, J.X. Yuan, and X. Wang, *J. Assoc. Off. Anal. Chem.,* **83**, 830 (2000).
110. J.F. Lawrence and C. Menard, *J. Chromatogr. B,* **696**, 291 (1997).
111. *Council Regulation (EEC) No. 2377/90 Annex I*, and amendments (1990).
112. *Code of Federal Regulations 21 Part 556, 1995*, and revisions (1995).
113. M.P. Marco, S.J. Gee, H.M. Cheng, Z.Y. Liang, and B.D. Hammock, *J. Agric. Food Chem.,* **41**, 423 (1993).
114. S.I. Wie and B.D. Hammock, *J. Agric. Food Chem.,* **30**, 949 (1982).
115. R. Jackman, *Anal. Chim. Acta,* **275**, 347 (1993).
116. P. Hammer and H. Heeschen, *Milchwissenschaft,* **50**, 513 (1995).
117. U. Albrecht, P. Hammer, and W. Heeschen, *Milchwissenschaft,* **51**, 209 (1996).
118. P. Hammer, H. Kirchhoff, and G. Hahn, *Anal. Chim. Acta,* **275**, 313 (1993).
119. C.A. Spinks, C.G. Schut, G.M. Wyatt, and C.M.R.A. Morgan, *Food Addit. Contam.,* **18**, 11 (2001).
120. L.H. Stanker, C. McKeown, B. Watkins, M. Vanderlaan, R. Ellis, and J. Rajan, *J. Agric. Food Chem.,* **41**, 1332 (1993).

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Validated immunoassay methods

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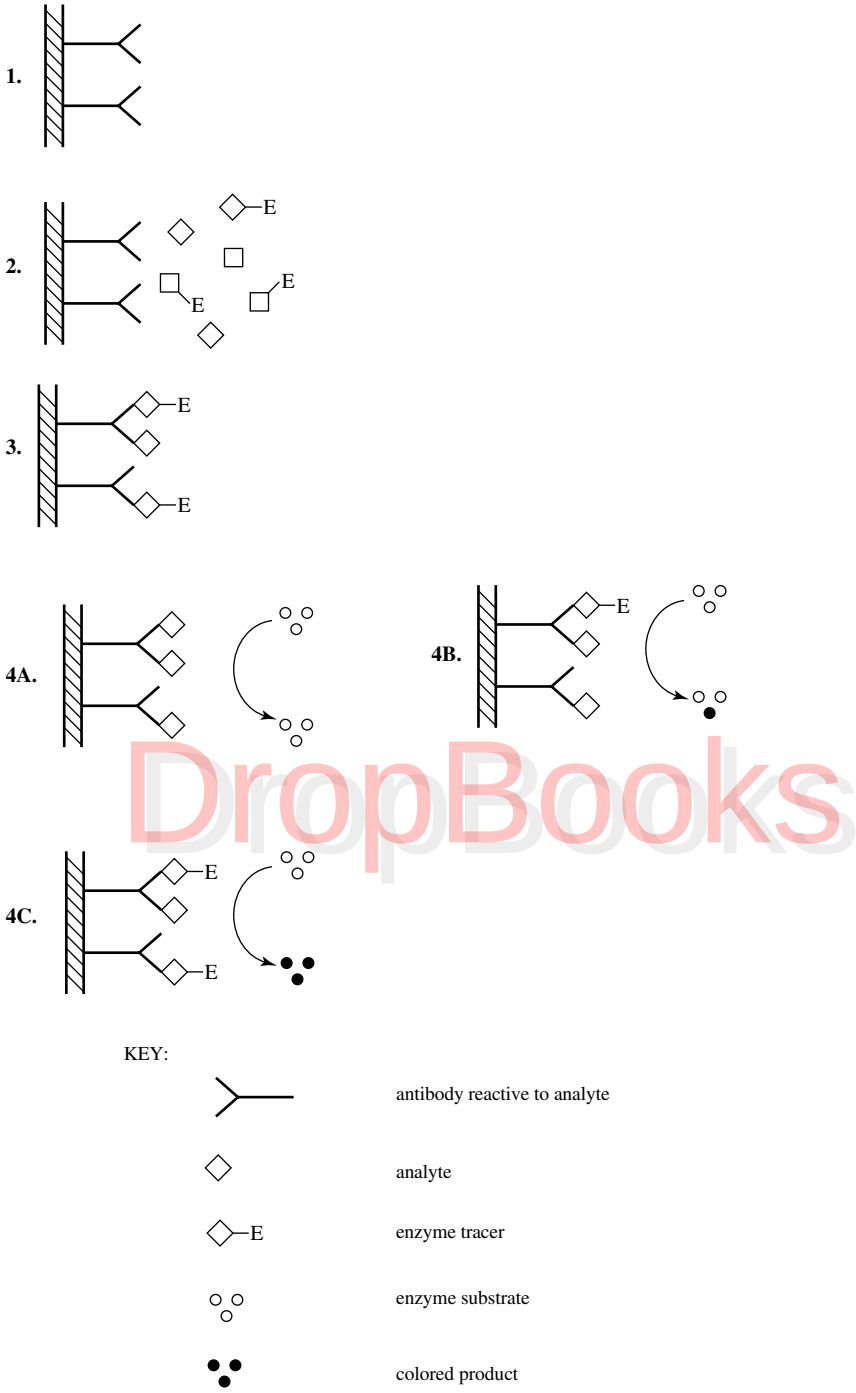
1 Introduction

Analytical methods for agrochemical residues intended for use as tolerance enforcement methods under US Environmental Protection Agency (EPA) guidelines must be validated according to specific requirements. The EPA has published data requirements for residue methods under Section 860.1340, with specifics for methods that employ chromatographic measurements for the determinative step. In August 1996, a revision of that section included a statement allowing the use of immunochemical methods.[1] To date, few immunoassay-based methods have been validated according to those guidelines and disclosed to the public or have been submitted to the EPA Office of Pesticide Programs (OPPTS) for use as tolerance enforcement methods. This article will focus on the development and validation of immunochemical methods to be used as enforcement methods. This will be accomplished by examining the theory and practice of enzyme immunoassays and comparing immunoassay- and chromatography-based methods. The requirements for tolerance enforcement will be discussed, and examples of immunochemical analytical methods validated according to these guidelines will illustrate the process. Examples will be drawn from the literature and from methods developed in this laboratory. Observations on the practical aspects of immunoassays will also be presented.

2 Enzyme immunoassays

The term 'immunoassay' is a generalized description of using antibodies for measurement purposes. In this article, 'immunoassay' will refer to a methodology depicted in Figure 1 called 'enzyme immunoassay' (EIA).[2] In this format, antibodies are coupled to a solid phase, usually cast from polystyrene, such as a culture tube or the well of a microtiter plate. The sample and an enzyme conjugated to a derivative of the analyte of interest are added to the reaction vessel. Analyte in the sample and in the enzyme conjugate compete for the constant, limited number of antibody binding sites. Binding of analyte in the sample prevents, or inhibits, the enzyme conjugate from binding. Hence, this part of the assay is often referred to as the 'inhibition step'. The reaction

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Figure 1 Schematic of an enzyme immunoassay. (1, 2) The test solution and enzyme conjugate are added to a tube or well pre-coated with anti-analyte antibodies. (3) After the inhibition step, the solid phase is washed, and only antibody-bound material is retained. (4A–C) Colorless substrate is added and is converted to a visible color in inverse proportion to the amount of analyte in the sample

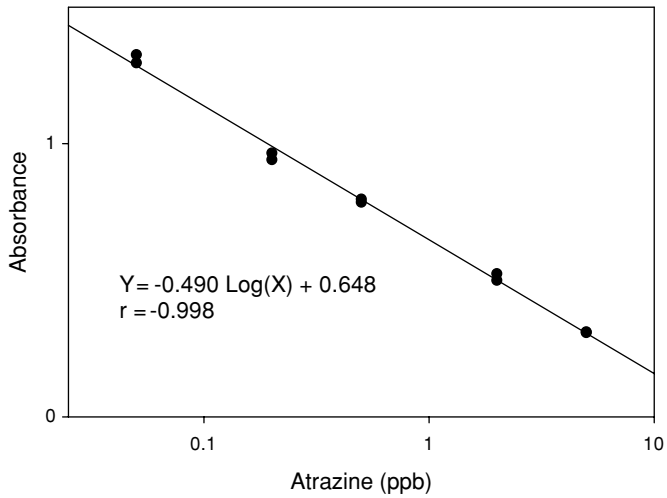


Figure 2 Typical enzyme immunoassay calibration curve illustrating the inversely proportional dose–response relationship

vessel is washed, removing all materials not bound to antibodies. Enzyme substrate is added. Substrate is colorless at the outset but is converted to a colored product by the bound enzyme. Generation of the colored product is terminated by acidification. Samples containing a high concentration of analyte bind little enzyme and produce weakly colored signals; the opposite is true for samples with a low concentration of analyte. As a consequence, calibration curves are inversely proportional to the concentration of the analyte (Figure 2).

EIAs are more desirable for the measurement of agrochemicals than enzyme-linked immunosorbent assays (ELISAs) for several reasons.[3] EIAs are easier to run, require minimal liquid transfers, and are completed in brief time frames, approximately 40 min for tube assays to 2.5 h for microtiter plate assays. In contrast, ELISAs are more complex, have many steps involving transfer of reagents, and require 6–8 h to complete. Most commercially available immunoassays utilize the EIA format.

2.1 Choice of tube or plate format

The choice of using tubes or plates depends on the expected sample load. If only a few samples are to be analyzed at one time, the tube format is ideal. Equipment requirements are minimal and quantitation can be carried out with a visible wavelength spectrophotometer. An analyst can become proficient with a tube assay after only a few practice trials, because only single-channel pipets are used to transfer reagents. The downside is that a single analytical set can accommodate only about a dozen samples, including controls and recovery samples. In contrast, 40 samples in duplicate can be analyzed on one microplate. The trade-off lies in a greater degree of skill required by the analyst and a much greater financial investment to conduct microplate assays. The analyst must be proficient with multichannel pipets for transferring small volumes of liquid, usually less than 0.20 mL. Microplates also require a dedicated photometer and special software. Regardless of the format selected, experience has shown that

whenever the absorbance data can be processed by commonly available spreadsheet software, the analyst should do so.

Several observations relative to plate assays should be noted. Antibody binding kinetics are proportional to temperature, and the long incubations associated with plate assays make microplates susceptible to variable binding should the room temperature fluctuate. This has been resolved in this laboratory by performing all incubations in a covered chamber such as under a cardboard box. Incubations in tube assays are so brief that temperature changes are not a concern. Performing plate incubations with shaking has been shown to increase precision of measurement. Finally, automated plate or strip washers are useful accessories for laboratories conducting analyses in the plate format.

Regardless of the format selected, samples and standards should always be mixed throughout an analytical set. In this manner, the first and last tube or well would contain a standard, with the remaining samples and standards intermixed. This serves as a check of the linearity of the assay response, because the calibration curve is based on standards spread throughout the set. Some commercial assays recommend running all standards prior to the samples. This approach cannot detect changes in pipetting rate or reagent handling over the entire set and is, therefore, not recommended.

Maintaining a moderate, consistent pipetting rhythm is the best way to ensure that all samples and standards are treated equally. This is easy to accomplish with tube assays, because relatively few samples can be analyzed per set. Microtiter plates present more of a challenge, because up to 96 wells may be utilized at the same time. One solution developed in this laboratory involves the use of a microtiter plate not coated with reagent – the reservoir plate.[4] An excess of all samples and standards is loaded into the reservoir plate. If 0.10 mL is needed for the inhibition step, for example, 0.15 or 0.20 mL of each solution is added to a pre-determined position in the reservoir plate; the excess amount simplifies the next pipetting step. The location of each sample and standard is identified on a plate layout sheet, a 'map' of the reservoir plate previously completed by the analyst (Figure 3). When the reservoir

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Plate Layout Sheet													
Plate ID:				Study No.:									
Analysis Date:				NB Ref.:									
Analyst:													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
A	0 ppb												**A**
B													**B**
C													**C**
D													**D**
E													**E**
F													**F**
G													**G**
H													**H**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
Comments													

Figure 3 Plate layout sheet

plate is completed, the analyst simultaneously transfers aliquots from all wells in a column of eight wells to the corresponding column in the antibody-coated plate using an eight-channel pipettor. This procedure is carried out across the reservoir plate. The enzyme conjugate or other reagent is added to the antibody-coated plate in a similar manner, except that the enzyme conjugate or reagent is pipetted from a commercially available reservoir specifically made for multichannel pipets. When liquid transfers are conducted in such a methodical, reproducible fashion, all antibody-coated wells are exposed to all reagents for the same length of time.

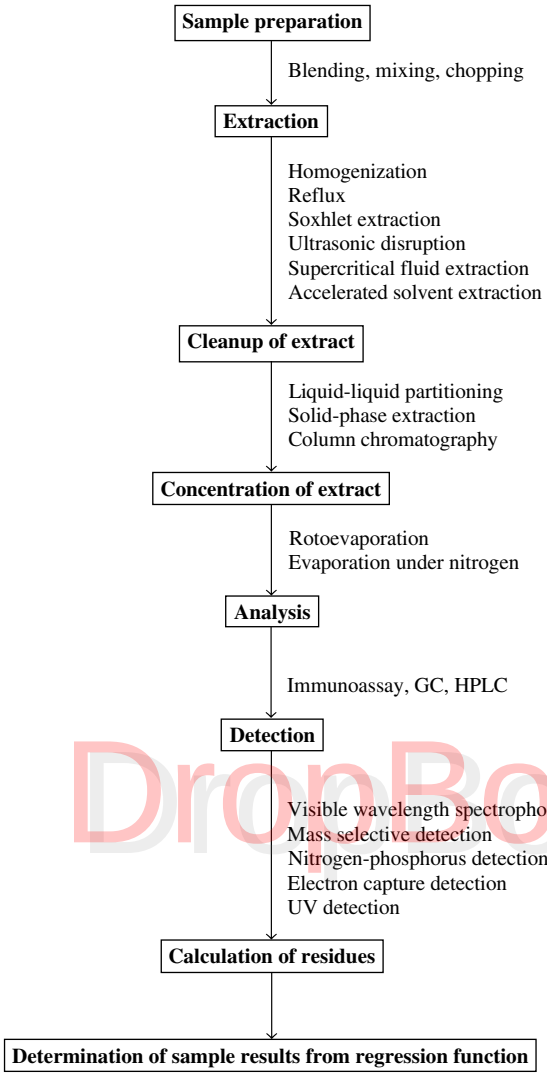
2.2 Calculation of residues

Immunochemists have applied a variety of mathematical models to immunoassay data.[5] Although curvilinear models such as the four-parameter logistic model[6] accurately describe the sigmoidial character of antibody–antigen interactions, two problems arise when this model is applied to the quantitation of residues. First, because the coefficients in the model are derived through a software-driven iterative process, verifying that the coefficients are correct may be difficult. The inability to verify that the software is operating properly is problematic from the viewpoint of Good Laboratory Practice (GLP),[7] which requires confirmation of software output. Second, the sigmoidial tails of the curve have such a shallow slope that they may not support a one-to-one relationship between analyte mass and detector response. Therefore, the analyst should restrict quantitation to the central linear portion of the curve where such a relationship is maintained. Using a straightforward log-linear plot (Figure 2) also simplifies the quantitation procedure, because regression packages are readily available in spreadsheet form, and results can be verified with a hand-held calculator.

2.3 Comparison with chromatography-based methods

Applying immunoassays to pesticide residue methods can be viewed as simply an adaptation of ‘classical’ residue technology. Indeed, immunoassay has been likened to merely a new detection system based on antibody recognition of the analyte. In essence, the immunochemist patterns the immunoassay-based method on the same set of overall procedures followed in a chromatography-based procedure. A generalized residue method is depicted in Figure 4 to visualize the process. A sub-sample is taken for analysis and extracted in an appropriate solvent, and an aliquot of the extract is prepared, or ‘cleaned up’, for analysis by isolating the analyte from compounds that would interfere with the measurement step. A fraction of the prepared aliquot is then subjected to analysis. The kinds of techniques performed at each step are suitable for most residue methods, regardless of measurement technique. Thus, extraction and cleanup techniques developed for chromatographic methods are readily transferable to immunoassay-based methods.

From an empirical viewpoint, the chief difference lies in the size of the aliquot that must be carried through the procedure. A typical sample size for a chromatographic method is 20 g. This is extracted, for illustration, in 100 mL of solvent. A volume of



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Figure 4 Flow chart of a typical agrochemical residue analysis

extract equivalent to half that mass (50 mL) is prepared for analysis, and the solvent is reduced to a few milliliters or less. As little as 2 μL of the concentrated extract may be injected for gas chromatography (GC), whereas 20–50 μL may be analyzed in high-performance liquid chromatography (HPLC) procedures. Calibration standards are typically in the micrograms per milliliter range. In contrast, an immunoassay method may require only a 1.0 g equiv. of extract, in this case 5 mL, to be cleaned up for analysis. Instead of reducing the aliquot volume, the extract prepared for analysis is usually restored to the original volume of the aliquot. A relatively large volume of extract is analyzed, approximately 100–500 μL, with standards in the nanograms per milliliter range.

There are several important differences between these analytical approaches that the analyst should recognize. First, chromatographic methods generally bring the concentrated extract into an organic solvent or organic–aqueous solution immediately prior to injection; organic solvents are mandated by GC systems, whereas reversed-phase HPLC columns utilize organic–aqueous solutions. Immunoassays, by comparison, are aqueous systems and can tolerate only limited amounts of organic solvents, generally up to 5% acetonitrile or 10% methanol in buffer or water; acetone is generally avoided, because this solvent precipitates protein. Poorly aqueous-soluble analytes can be brought into solution by amending buffers with surfactants, such as the Tween or Triton series (polyoxyethylene ethers), to a final concentration of 0.01 or 0.05%. Surfactants have also been added to wash solutions for more effective removal of hydrophobic compounds.

Second, each methodology has its own type of interferences. Interferences in a chromatographic system are viewed as compounds that elute near or at the retention time of the analyte of interest. Sample cleanup is directed at removing these compounds. In contrast, immunological interferences cause changes, positively or negatively, in the immunoassay response. These are regarded as specific and nonspecific. In the first case, compounds other than the desired target molecule are bound by antibodies. These 'cross-reactive' materials are chemicals of a similar size, shape, and charge as the target, such as simazine or propazine in an analysis for atrazine.[8] Chemicals of like structure may be difficult to remove from a sample because of their similar chemical properties. Cross-reactants may also share an immunoreactive moiety in their overall structure, such as the aromatic ring of alachlor in its ethanesulfonic acid metabolite.[9]

On the other hand, co-extractives or sample constituents that affect the assay response by some other means are lumped together as 'nonspecific' interferences. These were historically thought to interfere with antibody binding of analyte, but recent practice has shown that their effect upon the enzyme conjugate bears consideration. Horseradish peroxidase is frequently used to synthesize enzyme–analyte conjugates owing to its rapid turnover rate. However, the ubiquitous distribution of peroxidase isozymes in plants and animals suggests that molecules that control peroxidase activity are also widely distributed. The method developer should, therefore, be aware of the potential alteration of enzyme activity due to co-extraction of such compounds. Cinnamic acid derivatives,[10] conjugated linoleic acid,[11] D-mannose,[12] salicylic acid,[13] ascorbic acid,[14] and extracts of aged soybean seeds[15] have been cited as responsible for peroxidase inhibition. Khaziyev and Gul'ko[16] also found that humic acid inhibited peroxidase activity; humic and fulvic acids may be removed by passing an extract through strong anion-exchange solid-phase extraction (SPE) cartridges if the chemistry of the target molecule permits. Potential analytes carbaryl, dicofol, and dichlone were observed to stimulate peroxidase activity, whereas fenitrothion and dimethoate had a negative impact.[17] A. Krotsky (personal communication) found that aqueous extracts of control root crops gave strongly positive immunoassay responses. The problematic compounds were removed by back-partitioning the extract into methylene chloride. This case emphasizes the need to purify extracts of each substrate to the extent that control samples yield immunoassay responses similar to that of the blank, or zero standard.

Finally, a more subtle distinction lies in the manner in which the measurement step is carried out. This is accomplished in chromatographic methods by the

separation of sample components until the analyte of interest can be quantified in the absence of co-eluting peaks. No such separation occurs during an immunoassay. The immunological reagents are exposed to all constituents of the final extract during the inhibition step. In this sense, certain substrates may require more extensive cleanup for immunochemical analysis than for a chromatographic analysis. As stated above, the goal of the method developer should be to achieve similar immunoassay responses from the control substrate and the blank. Only in this manner can the analyst be assured that interferences associated with a given substrate have been adequately addressed.

Investigators have sometimes dealt with interferences by dilution or incorporating background interferences into the standards by preparing them in control extract. Dilution serves only to reduce the concentration of potential interferences, not remove them. Dilution also results in a corresponding decrease in assay sensitivity. Lucas *et al.*,[18] for example, diluted human urine 1 : 10 in buffer to reduce interfering substances in an analysis for atrazine mercapturate. While this step made immunoanalysis feasible, the dilution resulted in a 10-fold loss of sensitivity. In this laboratory, maintaining a low limit of quantitation (LOQ) (the lowest level of fortification for which recoveries in the range 70–120% can be obtained) was important. Organic extracts of urine were chromatographed on a diol SPE cartridge to achieve this goal. Concentration of the SPE eluate retained a 1.0 ng mL^{-1} LOQ and did not appreciably slow sample processing.[19] Workers have also added extracts of blank substrate to standard solutions to correct for substrate-specific interferences. Control substrate, however, may not always be available. Consequently, EPA requirements prohibit the use of control substrate as a means to address interferences in enforcement methods.[1]

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3 Requirements for validating a residue method

To understand how immunoassay-based analytical methods can be constructed to comply with tolerance enforcement requirements, a brief examination of those requirements is in order. This discussion is not intended to be comprehensive but to highlight aspects of special significance to immunoassay method development. The reader is urged to consult the literature[1,20,21] for further details.

A brief summary of EPA method requirements for tolerance enforcement methods is given in Table 1. Taken in total, these requirements ensure that the means to conduct the method are available to laboratories and that experimental evidence to establish method performance, on a substrate-by-substrate basis, is generated prior to analysis of samples and as part of each analytical set. Thus, an analyst who must generate data to support method performance in his or her hands can obtain whatever is required to reproduce the method.

These requirements have special implications with regard to immunoassay methods. First, the lack of commercial availability of reagents precludes preparing antibody-coated tubes or plates on-site, which may require knowledge of special skills. Commercial availability also ensures the analyst access to a reproducibly manufactured product. Therefore, the method must be based on an immunoassay that is a commercial product. Method developers may choose to introduce an in-house assay to the marketplace by partnering with a manufacturer, although this approach is costly and time-consuming.

Table 1 Summary of US EPA method requirements[a]

1	Method described in a stepwise fashion[b]
2	Commercial availability of reagents and equipment
3	Method must not be subject to substrate-related interferences (not require the use of blank substrate to correct for substrate-specific interferences)
4	Establish LOD and LOQ for each substrate
5	Control and recovery data for all substrates (blank substrate and blank substrate-fortified to LOQ)
6	Substrate/sub-sample must be fortified, not the extract
7	Recoveries of fortified samples in the range 70–120%
8	Specificity
9	Enforcement method to undergo independent laboratory validation study

[a]The reader should consult US EPA[1] for a complete description of the method requirements.
[b]A detailed outline of a written analytical method can be found in Mihaliak and Berberich.[20]

Second, the specificity of the method, or reactivity of the antibodies to other analytes that might be present in samples, must be thoroughly investigated. The analyst should determine what other agrochemicals might be present in a given substrate. These chemicals should be screened to ensure that the immunoassay does not generate false positive results. In most cases, this is likely to be a mechanical exercise given the selective nature of antibody binding. However, agrochemicals are often variations on common chemical themes such as the sulfonylurea (SU) class of herbicides. Development of an assay against one member of this class should include examination of antibody recognition of other SUs and their metabolites. For example, an immunoassay for triasulfuron was screened against 19 related parent SUs and degradates; only trace reactivity to three other SUs was observed.[4] As a result, the presence of other SUs in samples analyzed by the triasulfuron immunoassay is not a concern.

Third, the bulk of the items in Table 1 address method performance. These requirements must be satisfied on a substrate-by-substrate basis to address substrate-specific interferences. As discussed above, interferences are best dealt with by application of conventional sample preparation techniques; use of blank substrate to account for background interferences is not permitted. The analyst must establish a limit of detection (LOD), the lowest standard concentration that yields a signal that can be differentiated from background, and an LOQ (the reader is referred to Brady[5] for a discussion of different techniques used to determine the LOD for immunoassays). For example, analysis of a variety of corn fractions requires the generation of LOD and LOQ data for each fraction. Procedural recoveries must accompany each analytical set and be based on fresh fortification of substrate prior to extraction. Recovery samples serve to confirm that the extraction and cleanup procedures were conducted correctly for all samples in each set of analyses. Carrying control substrate through the analytical procedure is good practice if practicable.

Lastly, a laboratory not involved in the development process must validate the method. The independent laboratory validation study, or ruggedness trial, ensures that analysts unfamiliar with the method can successfully perform the method. The method developer should, therefore, strive to make all procedures as straightforward as possible to aid reproducibility of the method.

An additional requirement not noted in Table 1 is compliance with GLP.[7] These practices establish a paper trail for all procedures involved in the determination of residues. With regard to immunoassays, GLPs require calibration of measurement devices such as adjustable pipettors and dedicated spectrophotometers. Computer software output, as noted above, must be verified prior to use. This process can be simplified by limiting the application of specialized software to the operation of an instrument and carrying out the residue calculations in a broadly available spreadsheet such as Excel. On a positive note, in recent years, the software accompanying most microtiter plate readers has become generally easier to use and usually incorporates internal spreadsheets that are compatible with external systems.

3.1 Examples of validated immunoassay methods

The following methods serve as typical examples of immunoassay-based analytical methods applied to biomonitoring, environmental, and crop tissue analyses. Each method utilized a commercially available immunoassay kit that was combined with sample extraction and cleanup steps as part of an overall residue method. These methods can serve as models for resolution of similar problems.

Atrazine mercapurate [2-(L-cysteine-*N*-acetyl)-4-(ethylamino)-6-(isopropylamino)-*s*-triazine], a metabolite of atrazine in humans, was measured in urine as part of a study to assess the exposure of pesticide applicators and mixer/loaders to atrazine.[19] Aliquots (1.0-mL) were taken from urine samples collected at each void over a prescribed time period. Sodium chloride and HCl were added to the sample prior to liquid–liquid extraction with a solution of methylene chloride and ethyl acetate. The sample was extracted by vortex mixing and centrifugation to separate the phases. The organic layer was set aside, and the extraction was repeated twice. The combined organic fractions were dried over sodium sulfate and hexane. The dry organic extract was passed over a diol SPE column (Waters Milford, MA, USA), which retained the analyte. The analyte was eluted with alkaline ethanol. The eluate was evaporated to dryness and re-constituted in Tris–HCl buffer; duplicate aliquots of the buffered eluate were analyzed. The method utilized an EnviroGard atrazine plate kit (Strategic Diagnostics, Newark, DE, USA) designed to detect parent atrazine. The substantial cross-reactivity to the mercapturate formed the basis of the immunoassay measurement. The antibodies were more than four times as reactive to atrazine than to the mercapturate, but the diol cleanup step separated the polar degradation product from the nonpolar parent molecule. Measurements by GC failed to detect the parent molecule. Reactivity to the mercapturates of the chlorodegradates of atrazine was minimal. The method had an LOD of 0.50 ng mL^{-1} and an LOQ of 1.0 ng mL^{-1}. Procedural recoveries ranged from 86 to 112%.

A second example of a biomonitoring method is an analysis for atrazine in large-mouth bass plasma (Syngenta Crop Protection, unpublished data, 2002). This study presented the challenge of dealing with extremely small sample sizes, often less than 30 μL in volume. Aliquots of each sample, varying from 5 to 30 μL, were extracted directly on phenyl SPE cartridges (AnSys Technologies, Lake Forest, CA, USA). After dilution with water, the sample was passed through the cartridge. Atrazine

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residues were eluted in methylene chloride. The eluate was evaporated to dryness under nitrogen, and the residue was dissolved in water. Duplicate aliquots of the aqueous solution were analyzed. The method used the Beacon atrazine plate kit (Beacon Analytical Systems, Portland, ME, USA) that has a range of measurement of 0.05–5.0 ng mL^{-1}. As a result, the method LOD was 0.05 ng mL^{-1} . The LOQ was established at 0.10 ng mL^{-1}, and procedural recoveries averaged 95%. Cross-reactivity to other analytes was not a concern, because the samples were collected from fish exposed to atrazine in a controlled study.

An immunoassay-based method for the SU herbicide triasulfuron in soil and water is representative of a typical environmental method.[4] The EnviroGard triasulfuron plate kit (Strategic Diagnostics) was utilized for the determinative step. This assay selectively recognized triasulfuron among a variety of other SUs and their metabolites. Water and soil samples were collected from Kansas and North Dakota study sites, respectively. Water samples were analyzed directly without extraction. Soil samples were extracted in a methanol–phosphate buffer solution by vortex mixing and sonication. The extract was centrifuged, and a 1.0 g-equiv. of the supernatant was added to a C_8 SPE cartridge (Varian Sample Preparation Products, Harbor City, CA, USA). The extract was made acidic to reduce the water solubility of the analyte, which was retained on the column. Residues were eluted in methylene chloride, and the eluate was reduced to dryness. The residue was dissolved in a Tris–HCl buffer for immunoassay analysis. The immunoassay had an LOD of 0.05 ng mL^{-1}. The LOQ for water and soil samples was 0.05 and 0.10 ng mL^{-1}, respectively. Results of the immunoassay analyses compared favorably with chromatographic analyses of water (HPLC) and soil samples [high-performance liquid chromatography/mass spectrometry (HPLC/MS)].

The only published immunoassay method submitted to date to EPA OPPTS as an enforcement method for a range of substrates (water, sediment, crops, processed crop fractions, and animal tissues) is the spinosad method, developed by Young *et al.*[21] This method uses the spinosad RaPID Assay (Strategic Diagnostics) for determination of total spinosad residues (TSR). This discussion will be limited to crop and animal tissues, because the water and soil analyses are analogous to the triasulfuron method. The extraction, cleanup, and method parameters are summarized in Table 2.

Samples are extracted in acetonitrile or acetonitrile–water. The extracts are filtered or diluted prior to assay of beef tissues or milk. Extracts containing high concentrations of carbohydrates, such as apples, sorghum, and citrus produce, are passed through cyclohexyl SPE cartridges to remove the sugars. Residues in sorghum and apples are partitioned into dichloromethane and transferred into acetonitrile–water prior to SPE cleanup. Crop tissues containing high amounts of chlorophyll, including spinach and lettuce, undergo a novel treatment: sodium hypochlorite is added to these extracts to bleach out the so-called 'green material'. This is a unique contribution to cleanup procedures that should see wide application to a variety of crop tissues.

These authors noted the potential for the assay to underestimate the concentration of TSR due to decreased binding of metabolites relative to parent spinosad. However, the major residue found was parent spinosad, so underestimation of residues is not likely to be problematic. Overall, this method was validated in 34 matrices and showed excellent agreement with results obtained with a high-performance liquid chromatography/ultraviolet detection (HPLC/UV) method.[22]

Table 2 Spinosad method summary

Substrate	Sub-sample (g)	Extraction		Cleanup	LOD ($\mu g\,mL^{-1}$)	LOQ ($\mu g\,mL^{-1}$)
		Solvent[a]	Technique			
Beef tissue	20	ACN–H_2O (4 : 1)	Homogenization Reflux Filtration Evaporation	Dilution	0.003	0.01
Milk	5.0	ACN	Shaking Evaporation	Dilution	0.003	0.01
Apples, sorghum	5.0	ACN–H_2O (4 : 1)	Homogenization Shaking Centrifugation	Liquid–liquid partitioning with dichloromethane Evaporation Cyclohexyl SPE[b]	0.003	0.01
Citrus	5.0	ACN–H_2O (4 : 1)	Homogenization Shaking Centrifugation	Cyclohexyl SPE[b]	0.003	0.01
Other crops	5.0	ACN–H_2O (4 : 1)	Homogenization Shaking Centrifugation	Treatment with sodium hypochlorite[c]	0.003	0.01

[a]ACN = acetonitrile.
[b]Cyclohexyl SPE cleanup applied to citrus and sorghum samples only.
[c]Added to extracts of mustard greens, celery, head lettuce, leaf lettuce, spinach, and tobacco only.

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4 Conclusion

This article describes the theory behind enzyme immunoassays and the formats in which commercially available assays are constructed. Some observations pertinent to microtiter plate assays were presented. The manner by which data reduction is carried out was discussed, and comparisons with chromatography-based analytical methods were made. Interferences specific to immunoassays and suggestions to ameliorate their effects were presented. The requirements for validating a method according to US EPA guidelines were outlined. Finally, examples of immunoassay-based methods validated according to these guidelines for water, soil, biomonitoring, animal tissues, and crop tissues were discussed. It is hoped that this article will provide investigators with a real-world foundation upon which to build immunoassay-based methodologies for agrochemicals.

References

1. US EPA, Residue Chemistry Test Guidelines, OPPTS 860.1340, Residue Analytical Method, EPA 712-C-96-174, Office of Prevention, Pesticides and Toxic Substances, Environmental Protection Agency, Washington, DC (1996).
2. B.K. Van Weemen and A.H.W.M. Schuurs, *FEBS Lett.,* **24**, 77 (1972).
3. E. Engvall and P. Perlmann, *Immunochemistry,* **8**, 871 (1971).
4. J. Brady, J. Turner, and D. Skinner, *J. Agric. Food Chem.,* **43**, 2542 (1995).

5. J.F. Brady, *ACS Symp. Ser.,* **586**, 266 (1995).
6. D. Robard, in 'Ligand Assay,' ed. J. Langan and J.J. Clapp, Mason Publishing, New York, pp. 45–101 (1981).
7. US EPA, FIFRA, Good Laboratory Practice Standards, Final Rule (40 CFR Part 160 [OPP-300165A; FRL-3518-2]) RIN 2070-AB68, Environmental Protection Agency, Washington, DC (1989).
8. J. Brady, D. Tierney, J. McFarland, and M. Cheung, *J. Am. Water Works Assoc.,* **93**, 107 (2001).
9. D. Baker, R. Bushway, S. Adams, and C. Macomber, *Environ. Sci. Technol.,* **27**, 562 (1993).
10. R. Volpert, W. Osswald, and E.F. Elstner, *Phytochemistry,* **38**, 19 (1995).
11. H. Cantwell, R. Devery, M. O'Shea, and C. Stanton, *Lipids,* **34**, 833 (1999).
12. M. Soeiro, M. Paiva, H. Barbosa, M. Meirelles, and T. Araujo-Jorge, *Cell Struct. Funct.,* **24**, 139 (1999).
13. M. Ruffer, B. Steipe, and M. Zenk, *FEBS Lett.,* **377**, 175 (1995).
14. U. Takahama, *Plant Cell Physiol.,* **34**, 809 (1993).
15. J. Sung and C. Chiu, *Plant Sci. Limerick,* **110**(1), 45 (1995).
16. F.-Kh. Khaziyev and A. Ye. Gul'ko, *Sov. Soil Sci.,* **22**(4), 27 (1990).
17. L. Blagonravova and G. Nilov, Khim. Sel'sk. Khoz., **14**(11), 41 (1976).
18. A.D. Lucas, A.D. Jones, M.H. Goodrow, S.G. Saiz, C. Blewett, J.N. Seiber, and B.D. Hammock, *Chem. Res. Toxicol.,* **6**, 107 (1993).
19. J. Brady, J. Turner, M. Cheung, J. Kelly, D. King, and A. Alemanni, *ACS Symp. Ser.,* **683**, 131 (1998).
20. C. Mihaliak and S. Berberich, *ACS Symp. Ser.,* **586**, 288 (1995).
21. D. Young, C. Mihaliak, S. West, K. Hanselman, R. Collins, A. Phillips, and C. Robb, *J. Agric. Food Chem.,* **48**, 5146 (2000).
22. S. West, L.-T. Yeh, L. Turner, D. Schwedler, A. Thomas, and D. Duebelbeis, *J. Agric. Food Chem.,* **48**, 5131 (2000).

Advances in methods for pesticide residues in food

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1 Introduction

In the modern pesticide residues laboratory, analysts are under ever increasing pressure to (1) increase the range of pesticides which can be sought in a single analysis, (2) improve limits of detection, precision and quantitation, (3) increase confidence in the validity of residues data, (4) provide faster methods, (5) reduce the usage of hazardous solvents and (6) reduce the costs of analysis.

For example, in order to meet the demanding requirements of legislation such as the European Union (EU) Baby Food Directive (Directive 95/5/EC and subsequent revisions), analysts must improve on the scope and sensitivity of multi-residue methods of analysis. This Baby Food Directive, which became effective on 1 July 2002, limits residues of all pesticides to a maximum level of 0.01 mg kg^{-1}. There will also be a 'banned list' of pesticides, annexed to the Directive, which will not permit the use of certain pesticides on crops intended for use in baby food production. As a consequence, food manufacturers often require residue results for raw or primary ingredients within 24–48 h of sample receipt at the laboratory.

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Improvements in pesticide residue analysis have generally tended to follow, rather than lead, technological advances made in other fields of analytical chemistry. This is because residue analysis is a relatively small and specialized analytical chemistry market. In the early 1980s, the introduction of fused-silica capillary columns, as replacements for packed glass gas chromatography (GC) columns, provided the pesticide analyst with the additional resolving power necessary to separate a much large number of target analytes from co-extractives present in sample extracts. Capillary columns, with their lower flow rates of helium carrier gas, also paved the way for modestly priced, bench-top mass spectrometers which could be linked directly to gas chromatographs. The major advantages of these instruments were that they could be operated by nonspecialists and could provide more or less unequivocal data to confirm the identity of target analytes. Autosamplers improved the precision of injection, and also reduced costs per sample because they could be operated continuously day and

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night to improve the productivity of the laboratory. More advanced computer technology allowed full instrument control and, more importantly, much faster acquisition and data processing of the detector output from multi-component chromatographic separations.

During the last few years, miniaturization has become a dominant trend in the analysis of low-level contaminants in food and environmental samples. This has resulted in a significant reduction in the volume of hazardous and expensive solvents. Typical examples of miniaturization in sample preparation techniques are micro liquid/liquid extractions (in-vial) and solvent-free techniques such as solid-phase microextraction (SPME). Combined with state-of-the-art analytical instrumentation, this trend has resulted in faster analyses, higher sample throughputs and lower solvent consumption, whilst maintaining or even increasing assay sensitivity.

Most modern methods of analysis to determine pesticide residues in food commodities, whether a multi-residue method (MRM) or a single-residue method (SRM), can be broken down into three or four basic steps: sample processing, sample extraction, extract cleanup (optional) and instrumental determination.

2 Sample processing considerations

When samples of foods or crops are received at the laboratory, it is essential to take a representative portion for analysis. This is usually achieved by processing the sample into a more uniform state by cutting, chopping, mincing or milling. The lack of literature references in this area suggests that most laboratories have paid little attention to the resultant homogeneity of the samples or to the stability of pesticides using different sample processing techniques. Pettinati *et al.*[1] compared the use of a food chopper and bowl cutter for the preparation of meat samples and Lichon and James[2] evaluated a variety of milling and processing equipment for the homogenization of foodstuffs. However, neither considered the stability of analytes during processing.

Recent studies in our laboratory have clearly demonstrated that partial, and in some cases total, losses of pesticides such as chlorothalonil, dichlofluanid, ethoxyquin and tolylfluanid can occur when samples of fruits and vegetables are comminuted at ambient temperature. The extent of the loss is dependent on both the pesticide and the commodity, and can also vary between different varieties and even between different samples of the same variety. Losses of pesticides at the sample processing stage and (or) subsequent analytical steps will result in an underestimate of the residue level, with possible implications for both maximum residue level (MRL) compliance monitoring and consumer exposure. It is clearly desirable to develop and adopt sample processing procedures that eliminate, or at least minimize, residue losses. Arrhenius postulated that chemical reaction rates slow dramatically as the temperature is lowered. Enzymes are released when plant or animal cells are disrupted during processing, and these enzymes may be able to react with the pesticide residues also present in the sample. By reducing the temperature at which the samples are processed, these reactions can be slowed and losses of pesticides minimized. 'Cryogenic milling'[3,4] is a simple technique which can be employed to minimize losses. Fussell *et al.*[4] demonstrated that approximately 100 pesticides remained stable following freezing and subsequent cryogenic processing of apples. The pesticides included a number of

compounds that had previously been shown to degrade during processing at ambient temperature. Cryogenic milling is a technique that involves the sample being frozen at –20 °C before being disintegrated into a fine, friable powder using a bowl chopper in the presence of dry-ice (solid CO_2). The dry-ice ensures that the temperature is kept well below 0 °C during processing to ensure that the sample does not thaw. This technique has been successfully applied to many different pesticide–crop combinations, but there are a few examples, such as chlorothalonil in onions, where it is ineffective. In such cases, an additional technique (e.g., the addition of acid) may be required to minimize losses of residues (unpublished data).

A further advantage of cryogenic milling is that for certain commodities a more homogeneous sample is obtained. Cryogenic milling has been reported to give better disintegration of (1) the leaves in salad onions, (2) the woody basal plate in garlic and (3) the skins on tomatoes (unpublished data).

It has been known for many years that dithiocarbamates such as maneb or mancozeb are rapidly degraded when plant tissues and cells are disrupted.[5] In order to overcome this rapid degradation, fruit and vegetable samples are subjected to the minimum possible processing and only whole segments are cut out for analysis. Unless the pesticide residue is uniformly distributed throughout the sample (which it never is), the repeatability of consequent residue data will be very poor. Cryogenic milling may prove to be the solution to this problem.

3 Extraction procedures

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Conventional solvent extraction, where the sample is homogenized with a fixed volume (typically 50–100 mL) of solvent, remains the most widely used technique for solubilizing pesticide residues and isolating them from solid sample matrices. The most commonly used extraction solvents include acetone, acetonitrile, ethyl acetate, and methanol, which form the basis of multi-residue methods for the determination of pesticide residues in foods.[6–12] With the exception of the more polar pesticides, e.g., acephate and methamidophos, these solvents are equivalent in terms of their extraction efficiencies. A comprehensive EU study involving the determination of incurred residues (a total of 27 different pesticides in six representative food crops) demonstrated that there were no significant differences in the extraction efficiency of acetone or ethyl acetate for the majority of pesticides/commodity combinations tested.[13,14] In recent years there has been a concerted effort to reduce the volumes of solvents used in laboratories, especially chlorinated solvents such as chloroform and dichloromethane. Specht *et al.*[15] successfully modified their original multi-residue method[9] by replacing the dichloromethane used in the partition step with an ethyl acetate–cyclohexane mixture. Because of the need for laboratories to be more cost conscious and environmentally aware and to comply with more stringent regulations for the safe handling of chemicals, the use of alternative extraction techniques that significantly reduce the need for solvents have been developed. Techniques such as pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), and solid-phase extraction (SPE) not only reduce extraction and possibly also cleanup times, but also reduce waste solvent disposal costs and enable laboratories to make more efficient use of expensive laboratory storage space.

SFE can provide greater extraction selectivity, requires lower volumes of hazardous solvents than conventional solvent extraction and can be easily automated. Supercritical carbon dioxide is the most commonly used solvent for SFE because it is nontoxic, nonflammable, available at high purity and relatively low cost and can simply be vented into the atmosphere after the extraction has been completed. Supercritical carbon dioxide has the high solvating power of a liquid, but the low viscosity and high diffusivity of a gas. Therefore, it can penetrate into porous solid materials more effectively than solvents, resulting in a much faster mass transfer of the target analytes compared with conventional solvent extraction. The solvating power of the fluid is directly related to its density, which can be controlled as a function of both the temperature and pressure. The dissolution of interfering matrix co-extractives can be minimized by careful selection of the extraction conditions (density of the supercritical fluid, temperature, static time and flow rate) and by using selective adsorbents for trapping the target analytes. The extracts obtained using SFE may therefore be directly amenable to instrumental analysis without the need for any cleanup before the determination step. However, in order to improve the extraction efficiency of the more polar pesticides by increasing the fluid density or by adding a modifier, the technique becomes less selective and the levels of co-extractives are increased.

Despite its advantages, SFE is employed routinely in only a few pesticide laboratories, for the extraction of low-moisture samples such as grains, pulses, dried fruit and tea.[16] The technique requires specialized equipment because the extractions are performed at high pressure (45 psi) and elevated temperatures (around 60 °C) to maintain a CO_2 density of 0.85 g mL^{-1}. The analyte(s) may be trapped on C_{18} solid-phase material and eluted with a small volume (around 5 mL) of a polar solvent, such as acetonitrile.

SFE of fruits and vegetables[17,18] and meat products[19] has been reported, but the sample preparation techniques necessary to obtain reproducible results are extremely time consuming. Solid absorbents such as Hydromatrix, Extrelut[20] anhydrous magnesium sulfate[21] or absorbent polymers[22] are required to control the level of water in the sample for the extraction of the nonpolar pesticides. Without the addition of Hydromatrix, nonpolar pesticides cannot penetrate the water barrier between the sample particles and the supercritical CO_2. The sample is normally frozen and the addition of dry-ice may be required to reduce losses due to degradation and/or evaporation. Thorough reviews of the advantages and limitations of SFE in pesticide residues analysis have been published by Lehotay[23] and Stuart *et al.*,[24] and Yang *et al.*[25] included references to SFE in a specific review of the analysis of *N*-methylcarbamate pesticides.

Automated equipment designed to provide PLE, originally introduced by Dionex in 1995 and trade-named accelerated solvent extraction (ASE), has been available commercially for a number of years. It is gradually gaining popularity as a viable alternative to conventional solvent extraction. The system uses liquid solvents at elevated temperatures and pressures to improve the extraction kinetics that aid rapid extraction using low volumes of solvent. Typical operating conditions are pressure 1000–3000 psi, temperature 25 to –200 °C, sample size 1–30 g, extraction time 10–20 min and solvent volume 15–50 mL. Sand is mixed with the sample to assist the flow of solvent through the sample. For samples with a high water content, water-absorbing materials such as Hydromatrix (e.g., diatomaceous earth or sodium sulfate) must be

added. ASE is ideally suited to the analysis of dry foodstuffs because the increased penetration of solvent into the matrix and increased solubility of analytes at elevated pressure and temperature avoid the need for a 'water desorption' step.

Although the improved extraction kinetics also increase the concentration of co-extractives in the final extract, some degree of selectivity can be achieved by careful selection of the solvent or solvents used. Matrix co-extractives may be removed, or at least partially removed, by placing a suitable sorbent, such as alumina, at the exit of the extraction cell to remove lipid co-extractives. Excellent recoveries of both polar and nonpolar pesticides from a wide range of foodstuffs have been reported.[26] Specific applications include organophosphorus[27] and *N*-methylcarbamate[28] pesticides.

Weichbrodt *et al.*[29] reported on the use of focused open-vessel microwave-assisted extraction (FOV-MAE) for the determination of organochlorine pesticides in high-moisture samples such as fish. The results were comparable to those with closed-vessel microwave-assisted extraction (CV-MAE) and ASE. The main advantage of FOV-MAE is that the use of Hydromatrix is unnecessary as the solvent mixture of ethyl acetate and cyclohexane allows the removal of water from the sample matrix via azeotropic distillation.

Solid phase extraction (SPE) is a very simple, rapid and reproducible cleanup technique that is now widely accepted as an alternative to the time-consuming liquid–liquid extractions. Additionally, SPE uses relatively small volumes of solvents, and is easy to automate. It is available in a number of different formats, including cartridges, disks, loose material, well plates or SPME using film-coated capillaries. SPE can be considered as an extraction technique when used for isolation and concentration or a cleanup technique when used to remove co-extractives from solvent extracts. The use of SPE for cleanup is discussed later.

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The concept of SPME was first introduced by Belardi and Pawliszyn[30] in 1989. A fiber (usually fused silica) which has been coated on the outside with a suitable polymer sorbent (e.g., polydimethylsiloxane) is dipped into the headspace above the sample or directly into the liquid sample. The pesticides are partitioned from the sample into the sorbent and an equilibrium between the gas or liquid and the sorbent is established. The analytes are thermally desorbed in a GC injector or liquid desorbed in a liquid chromatography (LC) injector. The autosampler has to be specially modified for SPME but otherwise the technique is simple to use, rapid, inexpensive and solvent free. Optimization of the procedure will involve the correct choice of phase, extraction time, ionic strength of the extraction step, temperature and the time and temperature of the desorption step. According to the chemical characteristics of the pesticides determined, the extraction efficiency is often influenced by the sample matrix and pH.

Although SPME was applied initially for the analysis of relatively volatile environmental pollutants in waters, rapid developments have enabled SPME to be successfully applied for the analysis of pesticides in water,[31–34] wine[35–37] and more complex food samples such as honey,[38] fruit juice and pears,[39] vegetables[40] and strawberries.[41,42] With food samples, most analysts recognize the need for some sample pretreatment in order to minimize matrix effects. The matrix can affect the SPME efficiency, resulting in a reduced recovery of pesticides. The most common method is simply to dilute the sample or sample extract with water. Simplício and Boas[39] comminuted pears in water prior to the determination of pesticides. Volante *et al.*[40] extracted over 100 pesticides

from a mixed vegetable matrix using two different SPME phases. Recoveries were increased by sonicating with 4 mL (10-g sample), prior to suspension in water and SPME extraction. Although the SPME was sensitive for the majority of pesticides, the recoveries were still low compared with conventional methods. Hu *et al.*[41] and later Wang *et al.*[42] blended and centrifuged strawberries before an aliquot of the supernatant was subjected to SPME on a polydimethylsiloxane (PDMS)-coated fiber for 45 min at room temperature. The extracted pesticides were then desorbed from the SPME fiber into a GC[41] or high-performance liquid chromatography (HPLC)[42] sample vial. For the HPLC method, the detection limits were shown to be at low-$\mu g\,kg^{-1}$ levels and the linear response covered the range from 0.05 to 2.0 $mg\,kg^{-1}$ with good repeatability [3–9% relative standard deviation (RSD)]. The method is completely solvent free and the analysis for many pesticides takes only 1 h. One perceived disadvantage of SPME is that production of fortified recovery values for fortification samples is not possible. Any measurement made from a spiked sample can be considered as a new point of the calibration curve. For accurate quantification, the pesticides would have to be distributed homogeneously between the pulp and supernatant. However, SPME is still a useful technique for the rapid qualitative analysis of perishable commodities.

Lord and Pawliszyn[43] developed a related technique called in-tube SPME in which analytes partition into a polymer coated on the inside of a fused-silica capillary. In automated SPME/HPLC the sample is injected directly into the SPME tube and the analyte is selectively eluted with either the mobile phase or a desorption solution of choice. A mixture of six phenylurea pesticides and eight carbamate pesticides was analyzed using this technique. Lee *et al.*[44] utilized a novel technique of diazomethane gas-phase methylation post-SPE for the determination of acidic herbicides in water, and Nilsson *et al.*[45] used SPME post-derivatization to extract benzyl ester herbicides. The successful analysis of volatile analytes[46] indicates a potential for the analysis of fumigant pesticides such as formaldehyde, methyl bromide and phosphine.

The development of new fiber coatings in the near future should further improve the specificity of SPME and overcome some of the observed matrix effects. Quantification by stable isotope dilution gas chromatography/mass spectrometry (GC/MS) may assist in improving analytical performance. Along with the possible application of micro LC and capillary LC columns to in-tube SPME, the development of novel derivatization methods and the potential for the analysis of fumigant pesticides, SPME appears to be a technique with a future in the analysis of pesticide residues in food.

To overcome the problems of relatively low sample capacity associated with SPME, a technique known as stir-bar sorptive extraction has been reported by Baltussen *et al.*[47] A glass-coated magnetic stir bar was coated with 50–100 μL of PDMS. Sample extraction was performed by placing the stir bar in the sample with subsequent stirring for 30–120 min. After extraction, the stir bar was removed and analytes were thermally desorbed at 150–300 °C for 5 min for GC, or liquid desorbed for LC. Qualitative analysis of organochlorine residues in wine has been reported using a commercially available product known as Twister.[48]

The use of SPE disks represents a relatively new SPE approach for the rapid and efficient isolation of pesticides from aqueous samples. Empore extraction disks have approximately 10 times the cross-sectional area of conventional SPE cartridges with comparable solvent mass (500 mg). The dense packing and uniform particle

distribution allow faster flow rates (100 mL min^{-1} using standard filtration apparatus) but eliminate any channelling, thus reducing the potential for breakthrough. Disks can reduce solvent consumption by up to 90% compared with liquid–liquid extraction. Laganà *et al*.[49] compared the efficiency of Carbograph-1 SPE cartridges with LiChrolut-EN cartridges and polystyrene–divinylbenzene copolymer Empore disks for the extraction of acidic herbicides from water. Veningerová *et al*.[50] reported that polystyrene–divinylbenzene copolymer Empore disks produced good recoveries and results comparable to those of conventional methods for chlorinated pesticides in water. Excellent recoveries for the extraction of a range of pesticides from acetone–water extracts of fruits and vegetables using C_{18} disks[51] and using a combination of sorbents[52] have been reported.

Solid-phase sorbents are also used in a technique known as matrix solid-phase dispersion (MSPD). MSPD is a patented process first reported in 1989 for conducting the simultaneous disruption and extraction of solid and semi-solid samples.[53] The technique is rapid and requires low volumes (ca. 10 mL) of solvents. One problem that has hindered further progress in pesticide residues analysis is the high ratio of sorbent to sample, typically 0.5–2 g of sorbent per 0.5 g of sample. This limits the sample size and creates problems with representative sub-sampling. It permits complete fractionation of the sample matrix components and also the ability to elute selectively a single compound or class of compounds from the same sample. Excellent reviews of the practical and theoretical aspects of MSPD[54] and applications in food analysis were presented by Barker.[55] Torres *et al*.[56] reported the use of MSPD for the extraction of 18 pesticides from oranges. The sample (0.5 g) was blended with 0.5 g of C_{18} material and the mixture was loaded on to a column containing silica (0.5 g). The pesticides were eluted with 10 mL of ethyl acetate and the eluate was concentrated prior to analysis by GC. Recoveries ranged from 67 to 102% and were comparable to those with classical methods. Scibaldi *et al*.[57] reported the use of a diatomaceous earth-assisted procedure for the determination of more than 90 pesticides using GC with electron capture detection (ECD) and nitrogen–phosphorus detection (NPD) without the need for further cleanup.

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A relatively new SPE technique using a molecularly imprinted polymer (MIP) is gaining in popularity for the extraction of single-class or single-pesticide residues. MIPs possess high selectivity and sensitivity for low molecular mass components. The synthesis of MIPs is a relatively straightforward and inexpensive procedure, as outlined by Ensing *et al*.[58] A prearrangement of template molecules (pesticides) and functional monomers in solution prior to cross-linking polymerization preferably at low temperature generates a highly cross-linked polymer network. The removal of the template results in the formation/exposure of cavities in the polymer, which in the shape and spatial arrangement of functional groups are complementary to the template pesticide molecule. The use of MIP SPE has been reported for the extraction of pesticides from aqueous samples including terbumeton,[59] phenoxy acids[60] and triazines.[61,62] One of the limitations of MIPs is the nonspecific adsorption of a range of components in addition to the analytes of interest.

Rejeb *et al*.[63] described the development and characterization of immunoaffinity columns for the selective extraction of thifluzamide in peanuts. Efficient recovery was achieved using a simple elution profile requiring only 4 mL of methanol–water. De Jager and Andrews[64] described a novel fast screening method for organochlorine

residues in water. The relatively new technique of solvent microextraction (SME) was used to extract and preconcentrate the pesticides into a single drop of n-hexane. Using fast GC conditions, extraction and separation of 10 pesticides were achieved in 9 min. Capiello *et al.*[65] employed a range of pesticides to evaluate a micro SPE method for sample introduction in capillary liquid chromatography/mass spectrometry (LC/MS). Pesticides were concentrated at a high flow rate for fast trapping on a 2-cm packed capillary column. The flow was then switched and the trapped components were eluted directly on to a longer analytical column. Similarly, Hartmann *et al.*[66] had previously reported the use of on-line trace enrichment and determination of pesticides in water using custom-made coated capillaries combined with tandem mass spectrometry (MS/MS).

There are a large number of literature references that refer the use of SPE cartridges for the extraction of pesticides from water. There are several comprehensive reviews of the use of SPE, including that by Soriano *et al.*,[67] who discussed the advantages and limitations of a number of sorbents for the analysis of carbamates. Hennion[68] reviewed the properties and uses of carbon based materials for extraction of a wide multiclass range of pesticides. Thorstensen *et al.*[69] described the use of a high-capacity cross-linked polystyrene-based polymer for the SPE of phenoxy acids and bentazone, and Tanabe *et al.*[70] reported the use of a styrene–divinylbenzene copolymer for the determination of 90 pesticides and related compounds in river water. SPE cartridges are also widely used for the cleanup of solvent extracts, as described below.

4 Cleanup procedures

It is often difficult to define where sample extraction ends and cleanup procedures begin. Sample extracts may be injected directly into a gas or liquid chromatograph in certain cases, but this will be dependent on the analyte, sample matrix, injection, separation and detection system, and the limit of determination (LOD) which is required. It is also more likely that matrix-matched calibration standards will be needed in order to obtain robust quantitative data if no cleanup steps are employed.

For the majority of foods, especially those containing high levels of chlorophyll, carotenoids, waxes or fats, a cleanup technique is usually used to minimize contamination of the analytical instruments, especially for GC. There are a number of cleanup techniques that can be employed based on partition, adsorption, ion exchange and size exclusion.

As previously mentioned, SPE is used extensively to remove co-extractives from solvent extracts prior to chromatographic analysis. There is a diverse range of solid phases available in cartridge format which can be selected for a wide range of pesticide–food type combinations. SPE using porous materials such as diatomaceous earth, Florisil, alumina and silica gel has not altered significantly over recent years. However, the introduction of columns containing silica-based chemically bonded sorbents is relatively new. Bonded-phase technology involves attaching the functional group through a silyl ether linkage which produces a material that is generally stable between pH 2 and 7.5. There are several classes of sorbents, nonpolar (e.g., octadecyl, C_{18}), polar (e.g., cyanopropyl, CN) and ion-exchange (e.g., trimethylaminopropyl,

SAX) and carbon-based SPE sorbents, each of which offers unique properties. More recently, water-wettable, high-capacity polymer materials with dual ion-exchange reversed-phase retention mechanisms that are stable over the full pH range (0–14) have been developed.[71,72] The pesticides can be selectively eluted from the SPE cartridge whilst the co-extractives are retained, or vice versa, to provide almost infinite possibilities for dealing with a wide range of pesticide–matrix combinations. SPE is now well established and higher sample throughputs have been made possible by the introduction of SPE in 96-well plate formats.[73,74]

The use of SPE with porous materials such as alumina, diatomaceous earth, Florisil and silica for the cleanup of fat-soluble organochlorine pesticides in fatty foods such as meat, fish, shellfish, milk and vegetable oils has been well documented.[75–79] The choice of elution solvents is critical because relatively small amounts of lipid in the final extract can cause rapid deterioration of GC capillary columns and also contaminate the gas chromatograph. A number of workers have used a porous material in tandem with C_{18} to effect an improved cleanup.[75–79] Di Mucchio employed a multi-cartridge system comprising Extrelut, silica and C_{18} to extract organophosphorus pesticides from oils and fatty extracts.[79] Relatively few literature applications include the pyrethroids, but Ramesh and Balasubramanian[80] reported a simple carbon-based SPE method for the analysis of pyrethroids in vegetable oil.

For an individual pesticide (e.g., carbendazim) or a limited class of pesticides (e.g., carbamates, benzoylphenylureas, pyrethroids[81]), it may be possible to optimize the SPE conditions so that the pesticide(s) are selectively retained on the cartridge. A wash step can be introduced to elute the matrix selectively, thus producing an extremely clean extract. For example, when cleaning up sample extracts for carbendazim analysis, a cation-exchange (SCX) cartridge may be used and the pH carefully controlled to retain the carbendazim firmly, whilst the co-extractives are washed to waste. The carbendazim residues can then be eluted from the column by adjusting the pH.

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MRMs for pesticides in fruits and vegetables have also successfully used multiple cartridges in tandem to improve the effectiveness of the cleanup. For example, Fillion *et al.*[82] employed an octadecyl SPE tube to remove the nonpolar co-extractives, followed by a carbon SPE cartridge tube coupled to an aminopropyl tube to remove plant pigments such as chlorophylls and carotenoids from sample extracts. Using acetonitrile–toluene (3 : 1), mixtures of more than 200 pesticides were eluted with satisfactory recoveries. Unfortunately, chlorinated aromatics, particularly hexachlorobenzene, have a high affinity for carbon and yielded poor recoveries. Schenck and Howard-King[83] achieved good recoveries for a wide range of pesticides using a combination of graphitized carbon black (GCB) and primary/secondary amine (PSA) with acetone–toluene (3 : 1) as elution solvent. Schenck and Lehotay[84] evaluated various combinations of SPE cartridges to minimize the matrix effects in the analysis of pesticides in food samples. They found that the combination of anion-exchange and carbon SPE reduced, but did not eliminate, the matrix enhancement effect. Cook *et al.*[85] used a combination of C_{18} and Florisil for the determination of 89 pesticides in fruits and vegetables. Molinari[86] described the use of Extrelut for the rapid determination of 14 organophosphate pesticides in vegetables.

Advances in autosampler technology have encouraged the development of fully automated SPE procedures that require minimal manual intervention. Automated

procedures for the determination of pesticides in fruit and vegetables include those described by Hiemstra *et al.*,[87] who used diol-bonded silica cartridges for the determination of benzimidazoles, Columbé *et al.*,[88] who described the determination of 20 pesticides in fruits using silica SPE, and Kaufmann,[89] who described the fully automated determination of 21 pesticides in wine using C_{18} SPE followed by GC/MS.

Obana *et al.*[90] reported a modified ethyl acetate extraction which used a super absorbent polymer instead of sodium sulfate to absorb water. Following cleanup by carbon-based SPE and/or gel permeation chromatography (GPC), recoveries in excess of 70% were achieved for the majority of the 107 pesticides of interest in asparagus, orange, potato and strawberry. The super absorbent polymers are now being incorporated into ASE procedures.

For multi-residue analyses, GPC remains a popular choice. Separation is based on steric exclusion and is largely independent of analyte polarity. It is applicable to the majority of pesticides, making it a useful component of MRMs, especially for the removal of oils and fats from food extracts. High-performance GPC columns (typically 350 mm × 21.2-mm i.d.) are based on cross-linked, rigid gels. Although relatively expensive, they tend to be more robust than the manual, slurry-packed columns of styrene–divinylbenzene copolymers (such as Bio Beads SX-3) and yield more reproducible results. GPC has remained popular because the columns can be prepared in dimensions to suit the capacity, solvent consumption and analysis cycle time requirements of specific applications. Pesticides are generally collected in a tight band following the elution of the larger co-extracted molecules, such as chlorophyll. The main disadvantage of GPC is the solvent consumption of 100 mL or more per sample. By adding an autosampler and fraction collector, GPC and high-performance GPC procedures may be fully automated.

Recent developments in injector and autosampler designs have led to the introduction of direct sample introduction (DSI) or difficult matrix introduction (DMI), which allow the direct analysis of crude extract via on-line cleanup. This technique is described in the next section. Since contaminants are not able to build up in the system, the need for the cleanup of crude extracts and for instrument maintenance, is reduced. Lehotay[91] reported the use of DSI in combination with MS/MS for multi-pesticide residue analysis in samples of mixed fruit and vegetables. DMI linked to gas chromatography/tandem mass spectrometry (GC/MS/MS) limits the scope of the analysis to target compound analysis and the need for manual changing of the liner limits sample throughput. Fussell and Nicholas[92] reported on the use of DMI for the multi-residue analysis of pesticides in lettuce and peas. Using crude ethyl acetate extracts, chromatographic interferences experienced with conventional analysis were eliminated and excellent linearity, recovery, repeatability and sensitivity were obtained for about 20 pesticides using GC/MS in the selected ion monitoring (SIM) mode. Current work in the authors' laboratory on the use of DMI with gas chromatography/time-of-flight mass spectrometry (GC/TOFMS) (full-scan mode) should allow for the rapid and efficient screening of a much larger number of pesticides, at high sensitivity (0.01 mg kg^{-1}), with a total solvent use of only 60 mL per sample. Grob *et al.*[93] reported on a technique called injector-internal headspace analysis for the determination of organophosphorus pesticides in edible oils, and Zehringer[94] reported on the use of laminar cup liners for the determination of a range of pesticides in milk and fatty fruits.

5 Instrumental techniques for detecting, identifying and quantifying pesticide residues in food

Universal and selective detectors, linked to GC or LC systems, have remained the predominant choice of analysts for the past two decades for the determination of pesticide residues in food. Although the introduction of 'bench-top' mass spectrometers has enabled analysts to produce more unequivocal residue data for most pesticides, in many laboratories the use of selective detection methods, such as flame photometric detection (FPD), electron capture detection (ECD) and alkali flame ionization detection (AFID) or nitrogen–phosphorus detection (NPD), continues. Many of the new technologies associated with the on-going development of instrumental methods are discussed. However, the main objective of this section is to describe modern techniques that have been demonstrated to be of use to the pesticide residue analyst.

5.1 GC

GC can be used to separate about 80% of all the pesticides that are in current use or of current interest to residue analysts. Compounds that are particularly thermally labile or polar are not amenable to GC analysis, and are best analyzed using LC techniques.

5.1.1 New GC column techniques

Nearly all GC separations are performed using capillary columns ranging in length from 10 to 60 m. Capillary GC columns have excellent resolving power for complex mixtures of pesticide residues from co-extractives in sample extracts. As the column length is increased, the number of theoretical plates available is also increased and the resolving power is improved. However, longer columns will result in longer chromatographic run times and, if large sample numbers are being analyzed, increased run times can be disadvantageous. There are several ways by which run times can be shortened without a significant loss of resolution using techniques such as 'fast' or 'flash' chromatography.

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5.1.2 Fast chromatography

Fast chromatography involves the use of narrow-bore columns (typically 0.1-mm i.d.) that will require higher inlet pressures compared with the conventional wide-bore capillary columns. These columns require detectors and computing systems capable of fast data acquisition. The main disadvantage is a much-reduced sample loading capacity. Advances in GC column technology, along with many of the GC-related techniques discussed below, were recently reviewed by Eiceman *et al.*[95]

5.1.3 Flash GC

Maštovská *et al.*[96] demonstrated the use of a 5-m GC capillary column surrounded by a tube of resistively heated steel to raise the column temperature more rapidly and uniformly than a conventional GC oven. They analyzed 15 organophosphorus

pesticides that commonly occur as detectable residues in food crops using the Thermedics Detection EZ Flash upgrade kit with an HP 5890 Series gas chromatograph fitted with a flame photometric detector. Rapid cooling of the column resulted in a chromatographic cycle time of less than one tenth of that with a 30-m column in a conventional GC oven. Other advantages included a higher signal-to-noise ratio because of the much narrower peak widths and significantly improved retention time repeatability. For complex mixtures, any loss of resolution resulting from the use of a much shorter capillary column can be circumvented by connection to a time-of-flight (TOF) mass spectrometer with spectrometric resolution of any peaks that co-elute. One disadvantage compared with conventional capillary columns is that it is not possible to cut off short sections of the column that become contaminated with nonvolatile compounds which are present in many sample extracts.

5.1.4 GC × GC

The fundamental concept of two-dimensional (2-D) GC is not a particularly new one, but a paucity of commercial investment appears to have restricted its development. In recent years 'heart cutting', in which only a portion of an eluting peak from a first GC column is isolated and submitted to a second column, has been largely overtaken by comprehensive GC × GC.[97] In this latter case, the entire chromatogram eluting from the primary column is submitted to a secondary column of different polarity in a separate oven. The resulting 2-D chromatogram has peaks scattered about a plane rather than along a line. The additional separating power may be particularly useful when analyzing food or environmental sample extracts which contain many co-extractives. Marriott and co-workers[98,99] have published a number of papers presenting examples of the use of GC × GC. In a recent review, Marriott[100] demonstrated the use of two GC columns and cryogenic trapping to separate a mixture of 17 organochlorine pesticides and offered some hope for the future use of this technique. Dallüge *et al.*[101] employed comprehensive two-dimensional gas chromatography with TOF mass spectrometric detection to determine 58 pesticides in food. A longitudinally modulated cryogenic system (LMCS) was constructed and utilized not only to improve separation, but also to increase sensitivity. Complete separation of target analytes may not be necessary if an MS detector is available, but for conventional detectors (FPD, ECD, NPD, etc.) overlap of two or more chromatographic peaks is a major problem.

5.1.5 Injection techniques

Numerous types of GC injectors have been manufactured over the past four decades. The most commonly used injection techniques have been reviewed and described by Grob,[102] who correctly states that analysts must fully understand the techniques before they can make the most appropriate choice for their particular application(s). For most GC capillary column applications, the split/splitless, programmed-temperature vaporization (PTV) and on-column injectors remain the most popular. However, over the last few years, technology has progressed rapidly to provide injectors that allow more of the sample extract on to the GC column without overloading it.

5.1.6 Direct sample injection and large-volume injection

Conventional GC injectors allow 1–5 μL of sample extracts to be injected on to a capillary GC column. Modern large volume injectors (LVIs) have recently been designed which allow injection volumes of up to 1000 μL. By increasing the amount of sample extract injected, the amount of target analyte reaching the detector is also increased and hence much greater sensitivity can be achieved. Recent LVI design has been aimed at producing a discriminatory system that allows raw sample extracts to be analyzed directly on-line, negating time-consuming and expensive cleanup procedures. Lehotay[91] used a DSI device to inject 40-μL volumes of sample extracts from fruits and vegetables on to the GC column. The extract was placed in a micro vial in the GC injection port and the initial temperature was kept low to evaporate the solvent slowly with the split vent open. The split vent was then closed and the temperature rapidly increased to volatilize the semi-volatile pesticides on to the GC liner and column. Thus the volatility range of the analytes entering the liner and column matched the volatility range amenable to the GC system, and the nonvolatile components of the extract remained in the micro vial. The injector was not automated and the micro vial had to be manually discarded and replaced before the next injection. More recently, an automated LVI (ATAS Optic) has been developed[103] and used for the multi-residue determination of pesticides in lettuce. This large-volume dirty matrix injector (LV-DMI) GC/MS procedure utilizes a glass micro vial into which an aliquot of the sample extract is placed and held in a fritted GC liner in the injector. The conditions are optimized to allow the maximum transmission of the pesticides and minimum transmission of sample co-extractives on to the column. The injection liner is automatically replaced after each injection and the micro vial is disposed to waste.

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5.1.7 Selective detectors

Selective detection methods such as ECD, FPD and NPD have been used for the last four decades to detect and quantify a wide range of pesticide residues in a variety of sample extracts. Over the years, the designs of the detectors have been improved, which has led to increased sensitivity and stability. However, the main problem of poor specificity remains, and in general they cannot provide unequivocal evidence of analyte identity. The most significant development occurred in the early 1990s when Amirav and co-workers[104,105] developed the pulsed flame photometric detector (PFPD). The PFPD can amplify emissions from heteroatoms, such as P and S, whilst excluding hydrocarbon background emission. Thus improved sensitivity and higher selectivity can be obtained using this detector compared with the conventional FPD. A number of papers have been published demonstrating the use of the PFPD in the analyses of residues of organophosphorus and carbamate pesticides in fruits and vegetables.[106–108]

5.1.8 GC/MS

Mass spectrometry (MS) has proven to be far superior to other forms of detection for the determination and confirmation of pesticide residues in food, because its

specificity is unsurpassed. Traditionally, MS was performed on very large and expensive high-resolution sector instruments operated by experienced specialists. The introduction of low-resolution (1 amu), low-cost, bench-top mass spectrometers in the early 1980s provided analysts with a robust analytical tool with a more universal range of application. Two types of bench-top mass spectrometers have predominated: the quadrupole or mass-selective detector (MSD) and the ion-trap detector (ITD). These instruments do not have to be operated by specialists and can be utilized routinely by residue analysts after limited training. The MSD is normally operated in the SIM mode to increase detection sensitivity, whereas the ITD is more suited to operate in the full-scan mode, as little or no increase in sensitivity is gained by using SIM. Both MSDs and ITDs are widely used in many laboratories for pesticide residue analyses, and the preferred choice of instrument can only be made after assessment of the performance for a particular application.

5.1.9 Electron ionization

By far the most popular technique for producing ions for mass spectrometric measurement is electron ionization (EI). As the analyte molecules are introduced into the mass spectrometer they are bombarded with energetic electrons in a region of low pressure. This fragments the molecules, producing positively charged ions, the heaviest being the molecular ion, M^+, following the loss of a single electron from each neutral molecule. Some molecules produce intense molecular ions, whereas other molecules produce weak or negligible molecular ions. Ideally, a few ions of high mass and high abundance may predominate in the resulting spectra. However, frequently many ions of low abundance, and often of low mass, may occur, which are of limited use. The nature of this spectral pattern will ultimately determine the degree of sensitivity and selectivity that can be achieved. EI remains the most widely used ionization technique for the MS detection and determination of pesticide residues in food and environmental samples.

5.1.10 Chemical ionization

A number of pesticides do not produce an abundance of characteristic ions when EI is used. Hence relatively low sensitivity will be experienced, particularly in the full-scan mode. One possible way of overcoming this lack of sensitivity is to use a much softer ionization process such as chemical ionization (CI), which involves the use of a reaction gas such as methane. The resulting fragmentation may give rise to more abundant base peaks that can be used for both characterization and quantiation purposes. However, there is a further choice as positive chemical ionization (PCI) or negative chemical ionization (NCI) can be utilised. Many systems can be programmed to change ionization mode during an analysis to achieve the highest response from each target analyte. Hernando *et al.*[109] used this switching technique, together with MS/MS (see Section 5.1.11), on an ITD to analyse a mixture of organochlorine and organophosphorus pesticides in a pepper sample.

5.1.11 GC/MS/MS

GC/MS/MS can be extremely useful when the selection of appropriate fragmentation ions is limited. Secondary transitions produced by SRM can often be used to produce additional evidence for confirmation purposes and to provide more accurate quantitative measurements. GC/MS/MS may also be useful when severe interference is experienced with the ions formed during GC/MS. Sheridan and Meola[110] analyzed more than 100 pesticides in fruits, vegetables and milk and found that, in general, the sensitivity of MS/MS equalled that of traditional selective GC detection methods, such as FPD. In addition to providing more selectivity, MS/MS may also allow additional fragment ions to be monitored, for confirmation purposes, than when using MS alone (with SIM). Lehotay[91] used the selectivity of GC/MS/MS to analyze fruit and vegetable extracts directly, without any cleanup, for 22 targeted pesticides. He demonstrated that GC/MS/MS, when used in conjunction with DSI for 'on-line' cleanup, is a reliable and rugged system that saves time and overcomes detection interferences.

5.1.12 Time-of-flight (TOF) mass spectrometry

TOF mass analyzers are available for use when coupled to both GC and LC systems. Unlike conventional MS, the TOF mass analyzer does not scan. The ions are pulsed into the analyzer, allowing very rapid acquisition rates of up to 500 spectra per second. Classical library searchable spectra can be produced using a standard EI source. There is no spectral skew so automated deconvolution of overlapping peaks is possible. TOF can provide greater spectral resolution and mass accuracy than conventional benchtop mass spectrometers and hence fewer characteristic ions may need to be selected for unequivocal confirmation of analyte identity. In the last few years, papers describing various applications of time-of-flight mass spectrometry (TOFMS) have begun to appear. Hirsch *et al.*[113] demonstrated how such a system was used, in conjunction with fast GC, to separate and determine residues of the phenoxy herbicides in surface water samples. They concluded that the analysis of the methyl esters of the acid herbicides was accelerated by a factor of 5–10 when using a narrow-bore column and TOFMS over conventional GC/MS. Vreuls *et al.*[114] demonstrated the use of TOF to determine picogram levels of organophoshorus insecticides and triazine herbicides in river water.

Montero *et al.*[115] demonstrated the use of laser desorption (LD) coupled with resonance-enhanced multi-photon ionization (REMPI) and TOFMS to determine carbendazim residues in peppers. Homogenization of the sample with a carrier solvent was aided by cooling with liquid nitrogen. The resultant homogeneous suspension (particle size 5–25 μm) was sprayed on to a rotating Pyrex disk. Carbendazim was desorbed from the disk using an 8 mJ pulse of energy from an Nd:YAG laser and ionized with energy (1–3 mJ) from a second laser. Detection limits for carbendazim using these desorption and ionization techniques linked to TOFMS were quoted as being in the low-ppb range.

Perhaps a combination of fast, multi-dimensional GC and TOFMS together with LD sample introduction techniques offers the way forward for multi-residue analyses of food and environmental samples over the next few years.

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5.2 LC

Polar or thermally labile compounds – many of the more modern pesticides fall into one or other of these categories – are not amenable to GC and therefore LC becomes the separation technique of choice. HPLC columns may be linked to a diode-array detector (DAD) or fluorescence detector if the target analyte(s) contain chromophores or fluorophores. When using a DAD, identification of the analyte(s) is based on the relative retention time and absorption wavelengths. Similarly, with fluorescence detection, retention time and emission and absorption wavelengths are used for identification purposes. Both can be subject to interference caused by co-extractives present in the sample extract(s) and therefore unequivocal confirmation of identity is seldom possible.

5.2.1 LC/MS

As with GC/MS, LC/MS offers the possibility of unequivocal confirmation of analyte identity and accurate quantiation. Similarly, both quadrupole and ion-trap instruments are commercially available. However, the responses of different analytes are extremely dependent on the type of interface used to remove the mobile phase and to introduce the target analytes into the mass spectrometer. For pesticide residue analyses, the most popular interfaces are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Both negative and positive ionization can be used as applicable to produce characteristically abundant ions.

In addition to forming the basis of single-residue methods, LC/MS can also be used for some multi-residue analyses, although the number of target analytes is usually fewer (up to 30) compared with GC/MS (up to 150). Numerous papers describing the determination of carbamate, benzamidazole, phenyurea and benzoylurea residues in fruits, vegetables and soil have appeared over the past decade. Hogendoorn and van Zoonen[111] presented a comprehensive review of the use of LC for pesticide residue analysis in food and environmental samples. They conclude that LC/MS is rapidly becoming a routine technique for trace levels of polar pesticides because cleanup procedures can be simplified, thus reducing time and cost requirements.

5.2.2 Liquid chromatography/tandem mass spectrometry (LC/MS/MS)

As with GC/MS/MS, LC/MS/MS can also be used when the selection of appropriate fragmentation ions is limited. LC/MS/MS is gradually replacing LC/MS, not just because of the greater selectivity, but also because instrument prices have fallen dramatically in last 2–3 years. The determination of chlormequat residues in pears, as described by Startin *et al.*,[112] provides a good example of the value of MS/MS. The positive ESI mass spectrum of chlormequat is characterized by ions at m/z 122 and 124, corresponding to ^{35}Cl and ^{37}Cl, respectively. Sample extracts are reported to often produce potentially interfering peaks at m/z 122, and to a lesser extent m/z 124. These interferences are considerably diminished using the SRM transition m/z $122 \rightarrow 58$ and virtually no interference is encountered for the transition m/z $124 \rightarrow 58$ (Figures 1 and 2).

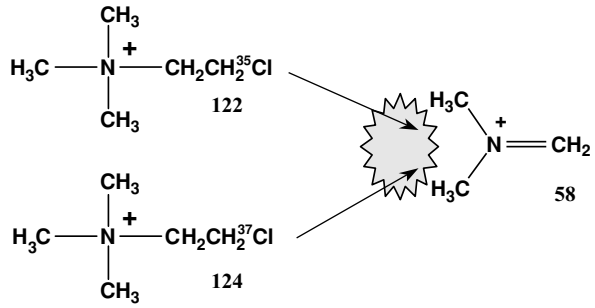


Figure 1 Selected reaction monitoring of the two primary chlormequat ions using MS/MS

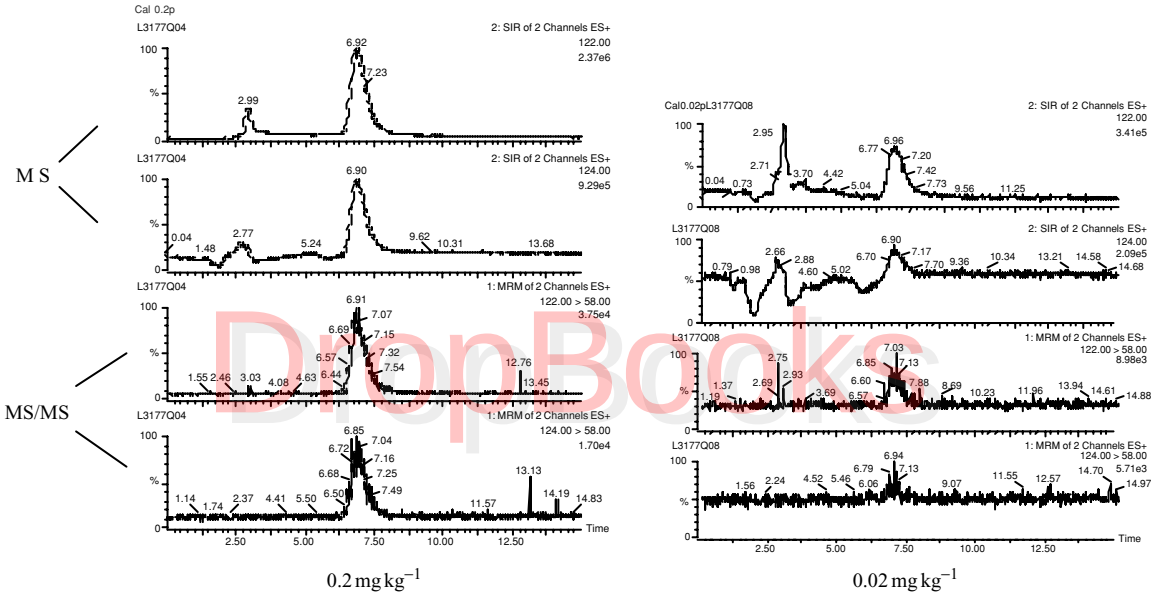


Figure 2 Primary and secondary ion chromatograms

5.3 Electrophoretic techniques

It is perhaps an indication of the limited success of electrophoretic techniques for the determination of pesticide residues at trace levels that although many papers and reviews on the subject have been published, very few laboratories involved in the routine analysis of residues rely on such techniques for their work. Electrophoretic techniques have suffered because of poor flexibility and sensitivities[116,117] compared with chromatographic techniques.

Capillary electrophoresis (CE) or capillary zone electrophoresis (CZE) is the technique most often employed in pesticide residue analysis. In its most basic form, free zone electrophoresis, a fused-silica capillary is filled with electrolyte (running buffer or background electrolyte). A potential is applied across the capillary and the cations

migrate to the cathode and anions to the anode. However, a unique feature of CE is the development of an electro-osmotic flow within the capillary that causes all ions, even anions, to migrate towards the cathode. In the time between writing his reviews in 1997[118] and 1999,[119] El Rassi demonstrated that advances had been made in the application of electrophoretic techniques. The most common form of CE is micellar electrokinetic chromatography (MEKC) and its application to the determination of pesticide residues in foods and commodities. In MEKC, a surfactant is added to the electrolyte. The surfactant can be anionic, e.g., the common use of sodium dodecyl sulfate (SDS), cationic or zwitterionic. Generally, the concentration of surfactant exceeds the critical micellar concentration (CMC) and micelles are formed, allowing the separation of neutral components interacting with the micelles. MEKC has been successfully applied to the separation of neutral pesticides. Wu *et al.*[120] described the separation of a mixture of carbamate pesticides using SDS in phosphate buffer. However, as in many publications in this area, separations were confined to relatively simple mixed standards or surrogate samples. In recent reviews by Eash and Bushway[121] and Menzinger *et al.*,[122] comprehensive tables listing publications on the application of electrophoretic techniques for pesticide analysis are cited. Of these, only a few are directly applicable to foods and commodities, the remainder of the methods cited being applications in the determination of residues in environmental samples, notably water. Table 1 presents a synopsis of methods for foods and commodities based on these reviews. Eash and Bushway[121] cite 53 applications, of which only five are applied to foods, and Menzinger *et al.*[122] cite 33 applications, of which only four are food related.

The availability of other chromatographic methods and the limitations of CE detection systems have caused CE to be used mainly for the determination of ionic herbicides in environmental samples. The technique has been successfully applied to herbicides such as glyphosate and its breakdown product, (aminomethyl) phosphonic acid,[132] and it has been extended to include glufosinate and its breakdown products using contactless conductivity detection.[133] Although subsequently applied to food materials, CE is far from being a routine tool. Methods for ionic species such as paraquat and diquat (bipyridinium herbicides), carboxylic acid herbicides, urea herbicides, triazines, sulfonylurea herbicides and plant growth regulators have all been extensively reported and reviewed.[5]

On-line sample-stacking techniques[134] and, more recently, the use of isotachophoresis have added to the potential benefits of CE by permitting the concentration of analyte in a large volume by exploiting the difference in the electric field between the dilute sample and system buffer. The electric field is much stronger in the dilute buffer–sample and hence analyte ions move faster until they reach the border with the separation buffer. At this point they slow down, causing the analyte to concentrate as a sharp sample band at the interface.

The principal limitation in the use of electrophoretic techniques is the lack of availability of suitable detection systems for quantitative analysis and unequivocal identification of pesticide analytes. Traditionally, either ultraviolet/visible (UV/VIS) or fluorescence detection techniques have been used. However, as with chromatographic techniques, MS should be the detection system of choice. A brief comparison of the numbers of recent papers on the application of GC/MS and LC/MS with capillary electrophoresis/mass spectrometery (CE/MS) demonstrates that interfaces between CE

Table 1 The use of electrophoresis in the determination of pesticide residues in foods

Analyte matrix	Mode	Detection	LOD	Buffer	Pesticides identified	Reference
Potatoes	CZE	UV	0.01 mg kg^{-1}	0.1 M phosphate, pH 2.5	Paraquat, diquat	123
Potatoes	CZE	DAD UV	0.4–0.5 mg kg^{-1}	100 mM phosphate, 10% acetonitrile	Paraquat, diquat	124
Cereals	MEKC	UV	0.02–0.04 mg kg^{-1}	50 mM SDS, 25 mM sodium phosphate (monobasic), pH 6.15	Metsulfuron-methyl, thifensulfuron-methyl, chlorsulfuron, rimsulfuron, tribenuron-methyl	125
Milk	Isotachophoresis	Conductivity	2 ng	40% methanol, 10 mM sodium acetate pH 4.8, 0.2% hydroxy-cellulose (leading electrolyte), 40% methanol, 20 mM acetic acid (terminating electrolyte)	Prometryne, desmetryne, terbutryne, atrazine (OH metabolites), simazine (OH metabolites)	126
Potatoes, onions	MEKC	UV	2 mg kg^{-1}	10 mM sodium phosphate, 40 mM cholic acid, pH 7	Maleic hydrazide	127
'Leaves'	CZE	UV	—	10 mM phosphate, 6 mM borate, 25% acetonitrile, 50 mM SDS	Chlorpyrifos	128
Soybeans	CZE	UV	11–85 µg kg^{-1}	50 mM acetate	Acifluorfen, 2,4-D, bentazone, thifensulfuron-methyl, chlorimuron-ethyl, imazaquin	129
Sugar cane, cereal	MEKC	UV	0.6 pg	50 mM borate, pH 8.3, 25 mM SDS	Dicamba	130
Wheat (milled)	CZE	UV	2 mg mL^{-1}	10 mM phthalate pH 7.5, 0.5 mM TTAB	Glyphosate, AMPA	131

AMPA = aminomethylphosphoric acid, CZE = capillary zone electrophoresis, DAD = diode array detector, MEKC = micellar electrokinetic chromatography, SDS = sodium dodecyl sulfate, TTAB = tetradecyltrimethylammonium bromide, UV = ultraviolet.

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buffer systems and MS ion sources still need much more development.[135,136] The use of nonaqueous CE systems will facilitate coupling with MS. For example, lipophilic pesticides[137] and sulfonylurea herbicides[138] have been successfully separated using acetic acid in methanol and acetate buffer in acetonitrile, respectively.

As concluded by Menzinger *et al.*,[122] CE will only fully augment chromatographic techniques in the routine determination of pesticide residues when software becomes capable of compensating for the intrinsic variability in analyte migration times. This

can be effected by, for example, changes in electroosmotic flow between individual capillaries by basing characterizations on effective mobility (μ_{eff} scale).[139]

5.4 Immunochemical and biosensor techniques

Like electrophoresis, the use of immunochemical techniques in the analysis of pesticide residues has yet to become as commonplace as perhaps it was once thought it would. The argument was that because conventional analytical methods were both costly and time consuming, the availability of immunologically based screening or pre-screening techniques would permit field testing or pre-screening of large numbers of samples prior to conventional analysis of positive samples. Pressures to reduce the unit costs of analysis and to increase the coverage of national pesticide residue monitoring programs have placed particular emphasis on the latter. The major problem is that 'pesticides' are not a homologous series of chemicals which can be tested as an entity or using a single biological activity, but are a diverse collection of complex, individual molecules with an array of physical and chemical properties.

Several comprehensive reviews of the development and use of immunochemical methods for the determination of pesticides have been written. Basic methodologies were explained by Hock *et al.*,[140] while Hennion and Barcelo[141] and, recently, Mallet *et al.*[142] highlighted the need for sensitive, easy-to-use, robust and cost-effective methods of analysis and suggested that although there are drawbacks, immunochemical methods offer one possible route to achieving these goals. Although the focus of these reviews is environmental analysis, the issues of maximizing assay sensitivity and ease of use and minimizing cross-reactivity with nontarget compounds are of equal importance in the determination of residues in food. For example, linking immunochemical methods with optical detection based on chemiluminescence and fluorescence[132] provides a route to greater sensitivity and, in some instances, greater selectivity, thus providing the basis for biosensors in rapid determination techniques.

Enzyme-linked immunosorbent assay (ELISA) techniques have been employed for over 20 years. For example, an ELISA based on monoclonal antibodies has been applied to the determination imidacloprid and acetamiprid in fruits and vegetables.[135] The assay, using a direct competitive format, has a sensitivity of 0.5–1.0 mg kg^{-1} (with recoveries of 80–120%) and low cross-reactivity, properties necessary for the analysis of a small range of specific residues in a limited range of matrices. The fungicide thiabendazole has been successfully determined in fruit juices using competitive, indirect ELISA.[34,136–138] The monoclonal antibody was capable of providing accurate determinations down to 0.5 mg kg^{-1}, about four times more sensitive than earlier assays. When applied to orange, grapefruit, apple, pear and banana juices the assay was demonstrated to work with both diluted juices and ethyl acetate extracts of juices. Other examples of pesticide ELISAs include the determination of the fungicide imazalil residues in citrus,[139] tetraconazole in fruit juices,[140] and carbaryl, carbofuran and methiocarb in strawberries and cucumbers.[141] Hennion and Barcelo[141] provide a comparison of ELISA with HPLC with fluorescence detection that serves to emphasize that although immunochemical methods are progressing to a point where they can rival chromatographic methods in sensitivity, immunoassays are best suited

to specific analyte–matrix combinations or narrow ranges where confirmation of the presence or absence of a residue is sought.

Biosensors may provide the basis for in-field analyses and real-time process analysis. However, biosensors are generally limited to the determination of a limited range of analytes in defined matrices. Enzyme-based biosensors, principally acetylcholinesterase (AChE) inhibition, have been successfully used in environmental analysis for residues of dichlorvos and paraoxon,[142] carbaryl[143] and carbofuran.[144] Immunochemically based biosensors may be the basis for the determination of pesticide residues in liquid samples, principally water and environmental samples, but also fruit juices.[145] The sensors can be linked to transducers, for example based on a piezoelectric effect, or flow-injection immunoanalysis (FII), in which the antigen–antibody reaction or column support is regenerated as part of an on-line procedure.[146] The way forward seems to be via a combination of routes. Chromatographic techniques, notably linked to mass detection systems, must continue to provide the mainstay for routine pesticide residue determinations. Coupled with the decreasing capital costs of GC, LC and MS instrumentation, the methods are increasingly rapid and provide unequivocal data across a wide range of determinants. Conversely, biosensors can provide the sensitivity and selectively necessary for the determination of narrow ranges of pesticide residues in specific matrices, for example in field testing a crop at the point of harvest, storage or sale as part of food-chain quality control programs. The next step in the development of biosensor technology may be to build upon the immobilization of enzymes on screen-printed sensors[147] to form arrays of sensors for different groups of pesticides within the same test system. It would be possible to link such an array to a visual means of recording the presence or absence of specific pesticides using pattern recognition. Likewise, the use of enzymes could be augmented in such arrays by the use of appropriate 'receptors' such as the use of Photosystem II (PS II) for the determination of triazine and phenylurea herbicides.[39,148] A similar approach has been used to monitor for the detection of estrogenic substances using the human estrogen receptor as the basis for a sensor.[149]

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Finally, the integration of biochemical or biosensor methods with 'conventional' chromatographic analyses should not be overlooked. For example, the use of immunoaffinity columns prior to chemiluminescence or the use of biosensor detection systems following the chromatographic step may provide useful solutions to specific analytical needs.

6 Future developments and trends

As the years progress, so the pace of new technological development seems to follow an exponential curve. It is impossible to predict all the changes that will occur, even in the near future, so we have selected four which we feel will have a significant impact on the work of pesticide residue analysts over the next 1–2 decades.

6.1 Silicon-based technologies

New silicon-based technologies will undoubtedly start to become commercially available in the near future. This will lead to miniaturized chromatographic systems and

the emergence of the 'lab on a chip', which will lead to more analyses being undertaken at the sample source rather than in the laboratory. Gas chromatographs based on micro-machining which integrate sub-100-μm i.d. columns and selective solid-state sensors will be used to undertake rapid, sensitive (zeptomole) analyses. However, new techniques for sampling and sample preparation, such as laser desorption (LD), will be needed if silicon-based technologies are to be fully utilized.

6.2 Biosensors

An alternative approach to the rapid detection and measurement of pesticide residues in situ, or on-line, is to use biosensors. A biosensor can be defined as a device consisting of a sensitive biological sensing element (immunosensor, enzyme sensor, etc.) associated with a transducer, which converts the biological signal into a measurable physical signal. A number of biosensors have already been developed for environmental monitoring, particularly of herbicides in groundwater. Undoubtedly, further biosensors will be developed which will be used to determine a wide range of pesticide residues in fresh fruits and vegetables directly on farms, in warehouses and supermarkets as well as in the laboratory.

6.3 Imprinted polymers

Jenkins *et al.*[150] reported on the development of sensors based on imprinted polymers directly polymerized on to a fiber-optic probe. A luminescent lanthanide (europium) was incorporated into the polymer to act as a signal transducer. Detection was then based on changes that occur in the spectrum when the pesticide is coordinated to Eu^{3+}. The combination of molecular imprinting and luminescence detection provided multiple criteria selectivity virtually to eliminate the possibility of false positive readings. Imprinted polymer sensors have been developed and used for the determination of glyphosate, diazinon and chlorpyrifos-methyl in water.

6.4 Analyses of chiral pesticides

The need to develop and use chiral chromatographic techniques to resolve racemates in pesticide residues will be driven by new hazard and risk assessments undertaken using data from differential metabolism studies. The molecular structures of many pesticides incorporate chiral centers and, in some cases, the activity differs between enantiomers. Consequently, in recent years manufacturers have introduced resolved enantiomers to provide pesticides of higher activity per unit mass applied. For example, the fungicide metalaxyl is a racemic mix of *R*- and *S*-enantiomers, both having the same mode of action but differing considerably in effectiveness. The *R*-enantiomer is the most effective and is marketed as a separate product metalaxyl-M. In future, it will not be satisfactory to rely on hazard/risk assessments based on data from metabolism studies of racemic mixes. The metabolism studies will need to be undertaken on one, or more, of the resolved enantiomers.

References

1. J.D. Pettinati, S.A. Ackerman, R.K. Jenkins, M.L. Happich, and J.G. Phillips, *J. Assoc. Off. Anal. Chem.,* **66**, 759 (1983).
2. M.J. Lichon and K.W. James, *J. AOAC Int.,* **73**, 820 (1990).
3. A.R.C. Hill, C.A. Harris, and A.G. Warburton, in 'Principles and Practices of Method Validation,' ed. A. Fajgeli and A. Ambrus, RSC Special Publication No. 256, Royal Society of Chemistry, Cambridge (2000).
4. R.J. Fussell, K. Jackson-Addie, S.L. Reynolds, and M. Wilson, *J. Agric. Food Chem.,* **50**, 441 (2002).
5. A.R.C. Hill, in 'Emerging Strategies for Pesticide Analysis,' ed. T. Cairns and J. Sherma, CRC Press, Boca Raton, FL, Chapt. 9, p. 217 (1992).
6. M.A. Luke, J.F. Froberg, and H.T. Masumoto, *J. AOAC Int.,* **58**, 1020 (1975).
7. S.M. Lee, M.L. Papathakis, H.M.C. Hunter, G.F. Hunter, and J.E. Carr, *Fresenius' J. Anal. Chem.,* **339**, 376 (1991).
8. P. Bottomley and P.G. Baker, *Analyst,* **109**, 85 (1984).
9. W. Specht and M. Tilkes, *Fresenius' J. Anal. Chem.,* **301**, 300 (1980).
10. CEN European Standard, prEN 12393-2–Part 2 (1996).
11. A. Andersson and H. Pålshden, *Fresenius' J. Anal. Chem.,* **339**, 365 (1991).
12. AOAC Methods PAM211 and PAM 231, Association of Official Analytical Chemists, Arlington, VA (1984).
13. S.L. Reynolds, R.J. Fussell, M. Caldow, S. Nawaz, C. Ebden, S. Lovell, and H. Diserens, EU Report 19306EN, European Commission, Brussels (2000).
14. S.L. Reynolds, R.J. Fussell, M. Caldow, S. Nawaz, C. Ebden, S. Lovell, and H. Diserens, EU Report 19306EN, European Commission, Brussels (2001).
15. W. Specht, S. Pelz, and W. Gilsbach, *Fresenius' J. Anal. Chem.,* **353**, 183 (1995).
16. B. Ohlin, A. Valverde, B. Declerq, K.N.T. Norman, G.G. Österdahhl, C. Elgerud, A. Aguilera, M. Roderiguez, M. Brontons, and S. Panton, Livsmedelsverkets Rapprort Nr. 10/2001, Livsmedelsverket, Uppsala (2001).
17. S.J. Lehotay and K.I. Eller, *J. AOAC Int.,* **78**, 821 (1995).
18. R. Stefani, M. Buzzi, and R. Grazzi, *J. Chromatogr. A,* **782**, 123 (1997).
19. R.K. Juhler, *Analyst,* **123**, 1551 (1998).
20. K.L. Pearse, V.C. Trenerry, and S. Were, *J. Agric. Food Chem.,* **45**, 153 (1997).
21. A. Valverde-Garcia, A.R. Fernandez-Alba, M. Contreas, and A. Aguera, *J. Agric. Food Chem.,* **44**, 1780 (1996).
22. M. Saka, K. Iijima, Y. Odanaka, and Y. Kato, *J. Pestic. Sci.,* **23**, 414 (1998).
23. S. Lehotay, *J. Chromatogr. A,* **785**, 289 (1997).
24. L.A. Stuart, J. MacLachlin, and A. McNaughtan, *Analyst,* **121**, 11 (1996).
25. S.S. Yang, A.I. Goldsmith, and I. Smtena, *J. Chromatogr. A,* **754**, 3 (1996).
26. Application Note **332**, Dionex, Sunnyvale, CA (1998).
27. H. Obana, K. Kikuchi, M. Okihashi, and S. Hori, *Analyst,* **122**, 217 (1997).
28. M. Okihashi, H. Obana, and S. Hori, *Analyst,* **123**, 711 (1998).
29. M. Weichbrodt, W. Vetter, and B. Luckas, *J. AOAC Int.,* **83**, 1334 (2000).
30. R.P. Belardi and J.B. Pawliszyn, *Water Pollut. Res. J. Can.,* **24**, 179 (1989).
31. A.A. Boyd-Boyland and J.B. Pawliszyn, *J. Chromatogr. A,* **704**, 163 (1995).
32. A.A. Boyd-Boyland, S. Magdic, and J.B. Pawliszyn, *Analyst,* **121**, 929 (1961).
33. J. Beltran, F.J. López, and F. Hernández *J. Chromatogr. A,* **885**, 389 (2000).
34. C. Aguilar, S. Penalver, E. Pocurull, F. Borrull, and R.M. Marce, *J. Chromatogr. A,* **795**, 105 (1998).
35. M. Vitali, M. Guidotti, R. Giovinazzo, and O. Cedrone, *J. Food Addit. Contam.,* **15**, 280 (1998).
36. L. Urruty and M. Montury, *J. Agric. Food Chem.,* **44**, 3871 (1996).
37. L. Urruty, M. Montury, M. Braci, J. Fournier, and J.-M. Dournel, *J. Agric. Food Chem.,* **45**, 1519 (1997).
38. J.J. Jiménez, J.L. Bernal, M.J. del Nozal, M.T. Martín, and A.L. Mayorga, *J. Chromatogr. A,* **829**, 269 (1998).

LoopBooks

39. A.L. Simplício and L.V. Boas, *J. Chromatogr. A,* **833**, 35 (1999).
40. M. Volante, M. Pontello, L. Valoti, M. Canttaneo, M. Bianchi, and L. Colzani, *Pestic. Manage. Sci.,* **56**, 618 (2000).
41. R. Hu, B. Hennion, L. Urruty, and M. Montury, *J. Food Addit. Contam.,* **16**, 111 (1999).
42. Z. Wang, B. Hennion, L. Urruty, and M. Montury, *J. Food Addit. Contam.,* **17**, 915 (2000).
43. H.L. Lord and J.B. Pawliszyn, *LC-GC Int.,* 776 (1998).
44. M. Lee, R. Lee, Y. Lin, C. Chen, and H. Bao-Huey, *Anal. Chem.,* **70**, 1963 (1998).
45. T. Nilsson, D. Baglio, I. Galdo-Miguez, J. Madsen, and S. Fachetti, *J. Chromatogr. A,* **826**, 211 (1998).
46. P.A. Marlos and J.B. Pawliszyn, *Anal. Chem.,* **70**, 2311 (1998).
47. E. Baltussen, P. Sandra, F. David, and C.J. Cramers, *J. Microcol. Sep.,* **11**, 737 (1999).
48. Application Note AN/2000/01-5, Gerstel, Mülheim a.d. Ruhr (2000).
49. A. Laganà, G. Fago, and A. Marino, *J. Chromatogr. A,* **796**, 309 (1998).
50. M. Veningerová, V. Prachar, J. Koračičová, and J. Uhnák, *J. Chromatogr. A,* **774**, 333 (1997).
51. J.A. Casanova, *J. AOAC Int.,* **79**, 936 (1996).
52. S.M. Price, J. Kellar, M. Carmean, J. Jabusch, M. Kennedy, M. Fellergy, D. Stover, and J. Warwick, Presented at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Chicago (1996).
53. S.A Barker, A.R. Long, and C.R. Short, *J. Chromatogr. A,* **475**, 63 (1989).
54. S.A. Barker, *J. Chromatogr. A,* **885**, 115 (2000).
55. S.A. Barker, *J. Chromatogr. A,* **880**, 63 (2000).
56. C.M. Torres, Y. Picó, M.J. Redondo, and J. Mañes, *J. Chromatogr. A,* **719**, 95 (1996).
57. F. Sicbaldi, A. Sarra, and G.L. Copeta, *J. Chromatogr. A,* **765**, 23 (1997).
58. K. Ensing, C. Berggren, and R.E. Majors, *LC-GC Eur.*, January, 16 (2002).
59. T.A. Sergeyeva, H. Matuschewski, S.A. Piletsky, J. Bendig, U. Schedler, and M. Ulbricht, *J. Chomatogr. A,* **907**, 89 (2001).
60. C. Baggiani, C. Giovannoli, L. Anfossi, and C. Tozzi, *J. Chromatogr. A,* **938**, 35 (2001).
61. E. Turiel, A. Martin-Esteban, P. Fernandez, C. Perez-Conde, and C. Camara, *Anal. Chem.,* **73**, 5133 (2001).
62. J. Matsui, K. Fujiwara, S. Ugata, and T. Takeuchi, *J. Chromatogr. A,* **889**, 25 (2000).
63. S.B. Rejeb, C. Cléroux, J.F. Lawrence, P.Y. Geay, S. Wu, and S. Stavinski, *Anal. Chim. Acta,* **432**, 193 (2001).
64. L.S. de Jager and A.R.J. Andrews, *Analyst,* **125**, 1943 (2000).
65. A. Cappiello, A. Berloni, G. Famiglini, F. Mangani, and P. Palma, *Anal. Chem.,* **73**, 298 (2001).
66. H. Hartmann, J. Burhenne, K. Müller, H.G. Frede, and M. Spiteller, *J. AOAC Int.,* **83**, 762 (2000).
67. J.M. Soriano, B. Jimenez, G. Font, and J.C. Molto, *Crit. Rev. Anal. Chem.,* **31**, 19 (2001).
68. M.-C. Hennion, *J. Chromatogr. A,* **885**, 73 (2000).
69. C.W. Thorstensen, O. Lode, and A.L. Christiansen, *J. Agric. Food Chem.,* **48**, 5829 (2000).
70. A. Tanabe, H. Mitobe, K. Kawata, and M. Sakai, *J. AOAC Int.,* **83**, 61 (2000).
71. E.S.P. Bouvier, P.C. Iraneta, U.D. Neue, P.D. McDonald, D.J. Phillips, M. Caparella, and Y.F. Cheng, *LC-GC Int.*, September, 35 (1998).
72. M. Early, *CAST*, January/February, 5 (2000).
73. D.D. Blevins and D.O. Hall, *LC-GC Int.*, September, 17 (1998).
74. D.A. Wells and R.E. Majors, *LC-GC Eur.*, March, 158 (2000).
75. F.J. Schenck, L. Calderon, and D.E. Saudarg, *J. AOAC Int.,* **79**, 1454 (1996).
76. A.M. Gillespie, S.L. Daly, D.M. Gilvydis, F. Schneider, and S.M. Walters, *J. AOAC Int.,* **78**, 431 (1995).
77. J.E. Quintanilla-López, R. Lebrón-Aguilar, and L.M. Polo-Díez, *J. Chromatogr. A,* **591**, 303 (1992).
78. R. Doong and C. Lee, *Analyst,* **124**, 1287 (1999).
79. A. Di Muccio, A. Ausili, L. Vergori, I. Camoni, R. Dommarco, L. Gambetti, A. Santillo, and F. Vergori, *Analyst,* **115**, 1167 (1990).
80. A. Ramesh and M. Balasubramanian, *Analyst,* **123**, 1799 (1998).
81. G.-F. Pang, Y.Z. Cao, C.-L. Fin, J.-J. Zhang, and X.-M. Li, *J. AOAC Int.,* **82**, 186 (1999).
82. J. Fillion, F. Sauvé, and J. Selwyn, *J. AOAC Int.,* **83**, 698 (2000).

83. F.J. Schenck and V. Howard-King, *Bull. Environ. Contam. Toxicol.,* **63**, 277 (1999).
84. F.J. Schenck and S.J. Lehotay, *J. Chromatogr. A,* **868**, 51 (2000).
85. J. Cook, M.P. Beckett, B. Reliford, W. Hammock, and M. Engel, *J. AOAC Int.,* **82**, 1419 (1999).
86. G.P. Molinari, S. Cavanna, and B. Ferroni, *J. Food Addit. Contam.,* **156**, 510 (1998).
87. M. Hiemstra, J.A. Joosten, and A. De Kok, *J. AOAC Int.,* **78**, 1267 (1995).
88. A. Columbé, S. Cardenas, M. Gallego, and M. Valcárcel, *J. Agric. Food Chem.,* **49**, 1109 (2001).
89. A. Kaufmann, *J. AOAC Int.,* **80**, 1302 (1997).
90. H. Obana, K. Akutsu, M. Okihashi, S. Kakimoto, and S. Hori, *Analyst,* **124**, 1159 (1999).
91. S.J. Lehotay, *J. AOAC Int.,* **83**, 680 (2000).
92. R.J. Fussell and D. Nicholas, *ATAS Chromatogr. Notes*, No. 82 (2002).
93. K. Grob, M. Biedermann, and A.M. Giuffre, *Z. Lebensm. Unters. Forsch.,* **198**, 325 (1994).
94. M. Zehringer, *J. Food Addit. Contam.,* **18**, 859 (2001).
95. G.A. Eiceman, H.H. Hill, Jr., and J. Gardea-Torresey, *Anal. Chem.,* **72**, 137R (2000).
96. K. Maštovská, J. Hajšlová, M. Godula, J. Křivánková, and V. Kocourek, *J. Chromatogr. A,* **907**, 235 (2000).
97. J.B. Phillips and J. Xu, *J. Chromatogr. A,* **703**, 327 (1995).
98. P. Marriot, R. Ong, and R. Shellie, *Am. Lab.*, August, 44 (2001).
99. T.T. Truong, P.J. Marriot, and N.A. Porter, *J. AOAC Int.,* **84**, 323 (2001).
100. P.J. Marriott, *Chem. Aust.,* **67**(11), 41 (2000).
101. J. Dallüge, M. van Rijn, J. Beens, R.J.J. Vreuls, and U.A. Th. Brinkman, *J. Chromatogr. A.,* **965**, 207 (2002).
102. K. Grob, *Anal. Chem.,* **66**, 1009A (1994).
103. R.J. Fussell and D. Nicholas, *ATAS Chromatogr. Notes*, No. 33 (2002).
104. E. Atar, S. Cheskis, and A. Amirav, *Anal. Chem.,* **63**, 2061 (1991).
105. A. Amirav and H. Jing, *Anal. Chem.,* **67**, 3305 (1995).
106. J.L. Martínez Vidal, F.J. Egea González, M. Martínez Galera, and M.L. Castro Cano, *J. Agric. Food Chem.,* **46**, 1440 (1998).
107. L.V. Podhorniak, J.F. Negron, and F.D. Griffith, Jr., *J. AOAC Int.,* **84**, 873 (2001).
108. M.E. Hernádez Torres, F.J. Egea González, M.L. Castro Cano, M. Moreno Frías, and J.L. Martínez Vidal, *J. Agric. Food Chem.,* **50**, 1172 (2002).
109. M.D. Hernando, A. Agüera, A.R. Fernández-Alba, L. Piedra, and M. Contreras, *Analyst,* **126**, 46 (2001).
110. R.S. Sheridan and J.R. Meola, *J. AOAC Int.,* **82**, 982 (1999).
111. E. Hogendoorn and P. van Zoonen, *J. Chromatogr. A,* **892**, 435 (2000).
112. J.R. Startin, S.J. Hird, M.D. Sykes, J.C. Taylor, and A.R.C. Hill, *Analyst,* **124**, 1011 (1999).
113. R. Hirsch, T.A. Ternes, I. Bobeldijk, and R.A. Weck, *Chimia,* **55**, 19 (2001).
114. R.J.J. Vreuls, V. Dallüge, and U.A. Th Brinkman, *J. Microcol. Sep.,* **11**, 663 (1999).
115. C. Montero, B. Bescós, J.M. Orea, and A. González Ureña, *Rev. Anal. Chem.,* **19**, 1 (2000).
116. J. Sherma, *J. AOAC Int.,* **82**, 561 (1999).
117. G. Guiochon, *Am. Lab.,* **30**, 14 (1998).
118. Z. El Rassi, *Electrophoresis,* **18**, 2465 (1997).
119. A. Karcher and Z. El Rassi, *Electrophoresis,* **20**, 3280 (1999).
120. Y.S. Wu, H.K. Lee, and S.F. Li, *J. Microcol. Sep.,* **10**, 529 (1998).
121. D.T. Eash and R.J. Bushway, *J. Chromatogr. A,* **880**, 281 (2000).
122. F. Menzinger, Ph. Schmitt-Kopplin, D. Freitag, and A. Kettrup, *J. Chromatogr. A,* **891**, 45 (2000).
123. Y. Wigfield, K.A. McCormack, and R. Grant, *J. Agric. Food Chem.,* **41**, 2315 (1993).
124. T. Perez-Ruiz, C. Martinez-Lozano, A. Sanz, and V. Thomas, *Chromatographia,* **43**, 468 (1996).
125. A.J. Krytinsky and D.M. Swineford, *J. AOAC Int.,* **78**, 1091 (1992).
126. L. Krivankovba, P. Bocek, J. Tekel, and J. Kovacicova, *Electrophoresis,* **10**, 731 (1993).
127. D.T. Kubilius and R.J. Bushway, *J. AOAC Int.,* **81**, 1109 (1998).
128. X. Guardino, J. Obiols, M.G. Rosell, A. Farran, and C. Serra, *J. Chromatogr. A,* **823**, 91 (1998).
129. S. Nemoto and S.J. Lehotay, *J. Agric. Food Chem.,* **46**, 2190 (1998).
130. F.M. Lancas, S.R. Rissato, and M.S. Galhiane, *Chromatographia,* **50**, 35 (1999).
131. M.G. Cikalo, D.M. Goodall, and W. Matthews, *J. Chromatogr. A,* **745**, 189 (1996).

DropBooks

132. M. Tomita, T. Okuyama, Y. Nigo, B. Uno, and S. Kawai, *J. Chromatogr. A,* **571**, 324 (1991).
133. L. Goodwin, M. Hanna, J.R. Startin, B.J. Keely, and D.M. Goodhall, *Analyst,* **127**, 204 (2002).
134. A. Farran, S. Ruiz, C. Serra, and M. Aguilar, *J. Chromatogr. A,* **737**, 109 (1996).
135. C.Y. Tsai, Y.R. Chen, and G.R. Her, *J. Chromatogr. A,* **813**, 379 (1998).
136. K. Otsuka, C.J. Smith, J. Grainger, J.R. Barr, D.G. Patterson, N. Tanake Jr., and S. Terabe, *J. Chromatogr. A,* **817**, 75 (1998).
137. Q. Yang, L.M. Benson, K.L. Johnson, and S. Naylor, *J. Biochem. Biophys. Methods,* **38**, 103 (1999).
138. W.H. Matchett, W. Winnik, and W.C. Brumley, *J. Capillary Electrophoresis,* **3**, 199 (1996).
139. Ph. Schmitt-Kopplin, A.V. Garmash, A.V. Kudryavtsev, I. Perminova, N. Hertkorn, D. Freitag, and A. Kettrup, *J. AOAC Int.,* **82**, 1594 (1999).
140. B. Hock, A. Dankwardt, K. Kramer, and A. Marx, *Anal. Chim. Acta,* **311**, 393 (1995).
141. M.-C. Hennion and D. Barcelo, *Anal. Chim. Acta,* **362**, 3 (1998).
142. E. Mallet, D. Barcelo, C. Barzen, G. Gaugglitz, and R. Abuknesha, *Trends Anal. Chem.,* **20**, 124 (2001).
143. B. Danielsson, I. Surugiu, A. Dzgoev, M. Mecklenberg, and K. Ramanathan, *Anal. Chim. Acta,* **462**, 227 (2001).
144. S. Wanatabe, I. Shigekazu, Y. Kamata, N. Omoda, T. Yamazaki, H. Munakata, T. Kaneko, and Y. Yuasa, *Anal. Chim. Acta,* **427**, 211 (2001).
145. R.J. Bushway, D.L. Brandon, A.H. Bates, L. Li, K.A. Larkin, and B.S. Young, *J. Agric. Food Chem.,* **43**, 1407 (1995).
146. R.J. Bushway, K. Larkin, and B. Perkins, *Food Agric. Immunol.,* **9**, 249 (1997).
147. A. Abad, J.J. Manclus, M.J. Moreno, and A. Montoya, *J. AOAC Int.,* **84**, 156 (2001).
148. E. Watanabe, S. Watanabe, S. Ito, M. Hayashi, T. Watanabe, Y. Yuasa, H. Nakazawa, *J. Agric. Food Chem.,* **48**, 5124 (2000).
149. S. Cairoli, A. Arnoldi, and A. Pagani, *J. Agric. Food Chem.,* **44**, 3849 (1996).
150. A.L. Jenkins, R. Yin, and J.L. Jensen, *Analyst,* **126**, 798 (2001).

Overview of analytical technologies available to regulatory laboratories for the determination of pesticide residues

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1 Introduction

The development of a robust analytical method is a complex issue. The residue analyst has available a vast array of techniques to assist in this task, but there are a number of basic rules that should be followed to produce a reliable method. The intention of this article is to provide the analyst with ideas from which a method can be constructed by considering each major component of the analytical method (sample preparation, extraction, sample cleanup, and the determinative step), and to suggest modern techniques that can be used to develop an effective and efficient overall approach. The latter portion emphasizes mass spectrometry (MS) since the current trend for pesticide residue methods is leading to MS becoming the method of choice for simultaneous quantitation and confirmation. This article also serves to update previous publications on similar topics by the authors.[1,2]

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It is a regulatory requirement that analytical methods be developed to determine residues of concern in crops, feed, and food commodities as well as environmental samples (air, soil, and water). Methods for crops, feed, and food commodities are required for enforcement purposes but are also needed for a variety of other purposes, such as gathering monitoring data for risk assessment. For nearly any purpose, the methods must be robust, that is, when used by different analysts in several laboratories, they should provide reproducibly similar results.

All aspects in the analytical process are equally important, and each step should be isolated in method development experiments and/or validation to ensure acceptable quality of results. A good way to evaluate robustness of a method is to alter parameters (e.g., solvent volumes, temperature, pH, sources of reagents) of each step to determine

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the effect. This will show the boundaries in the performance of the method to still achieve acceptable results and indicate critical procedures.

When developing or routinely using an analytical method, quality control (QC) fortifications can be added to each sample at critical points in the procedure to ensure that sensitive steps in the method were conducted properly and to pinpoint where problems occurred if results are less than satisfactory. For example, if the QC fortification samples for detection and cleanup were to show acceptable results in a batch of samples, but the extraction QC spike gave low recovery and/or high variability, then the analyst could modify instrument conditions or altering cleanup parameters immediately. Likewise, if the QC spike added just before analysis gives poor results, then instrument maintenance could be done and the samples merely re-analyzed rather than re-extracted.

When developing a new analytical method, the sequence of experiments should actually work backwards through the method. Analytical separation and detection parameters should be optimized first, otherwise extraction results could not be obtained. However, the overall analytical strategy actually begins with devising an adequate statistically representative sample collection scheme, proper sample handling protocols, and appropriate quality assurance (QA)/QC guidelines to meet the goals of the analysis. Then validation should be performed, and a list of factors that should be evaluated has been prepared for the validation of analytical method performance in individual laboratories.[3]

2 Sample preparation

Sample preparation consists of homogenization, extraction, and cleanup steps. In the case of multiresidue pesticide analysis, different approaches can have substantially different sample preparation procedures but may employ the same determinative steps. For example, in the case of soil analysis, the imidazolinone herbicides require extraction of the soil in 0.5 M NaOH solution, whereas for the sulfonylurea herbicides, 0.5 M NaOH solution would completely decompose the compounds. However, these two classes of compounds have the same determinative procedure. Some detection methods may permit fewer sample preparation steps, but in some cases the quality of the results or ruggedness of the method suffers when short cuts are attempted. For example, when MS is used, one pitfall is that one may automatically assume that all matrix effects are eliminated because of the specificity and selectivity of MS.

2.1 Extraction

The extraction procedure begins the process to separate the analytes from the matrix and present the material in a form that can be more easily analyzed. The type of extraction step that is used for a particular matrix depends on the nature of the matrix and analytes. There are two competing views in the extraction process among chemists. Some prefer to extract the analytes exhaustively from the matrix and rely on extensive cleanup to remove matrix co-extractives. Others prefer the 'just enough' extraction concept, in which the selectivity of the extraction process is honed as much as possible

Table 1 Properties of common solvents used in pesticide residue analysis at 20–25 °C

Solvent	Polarity index	Solubility in water (%, w/w)	Density ($g\,mL^{-1}$)	Viscosity ($mN\,s\,m^{-2}$)	Expansion volume[a]	Boiling point (°C)
Water	10.2	—	0.998	1.00	1416	100.0
Acetonitrile	5.8	100	0.786	0.37	488	81.6
Acetone	5.1	100	0.791	0.32	348	56.2
Methanol	5.1	100	0.792	0.60	632	64.6
Ethanol	5.2	100	0.789	1.20	438	78.4
Ethyl acetate	4.4	8.7	0.902	0.45	261	77.2
2-Propanol	3.9	100	0.785	1.76	334	82.4
Dichloromethane	3.1	1.6	1.33	0.44	399	40.7
Diethyl ether	2.8	6.9	0.713	0.24	246	34.6
Toluene	2.4	0.052	0.866	0.52	240	110.8
Cyclohexane	0.2	0.006	0.789	0.98	268	80.7
Isooctane	0.1	0.002	0.692	0.50	155	99.0
n-Hexane	0.0	0.014	0.660	0.32	196	69.0

[a]At 250 °C and 10 psi (gauge) pressure, 1 unit liquid volume will become the given value in the same units in the gas phase.

to achieve high recoveries of the analyte(s) but as little matrix co-extractives as possible. The latter approach saves time, labor, and expense, but the former approach may give higher recoveries in a wider variety of matrices.

The most common application in chemical residue analysis concerns the extraction of a solid sample using a liquid. A variety of liquid solvents are readily available to provide a medium for easy homogenization in a blending or shaking device. Table 1 gives, in order of decreasing polarity, a comparison of important considerations in choosing common liquids for use in pesticide residue methods. The boiling point is a measure of volatility of the solvent and gives an idea of how easily and quickly the solvent can be evaporated in solvent concentration steps. Viscosity and expansion volumes are indicators of the potential usefulness of the solvents in high-performance liquid chromatography (HPLC) and gas chromatography (GC), respectively [of course, other parameters such as polarity, ultraviolet (UV) cutoff wavelength, and phase compatibility may be very important depending on the analysis conditions]. Density indicates if the solvent will form the upper or lower layer in liquid–liquid partitioning procedures (salts or co-solvents can be added to form a water/solvent partition in the case of water-miscible solvents). Solubility in water indicates how well liquid–liquid partitioning can be done for pesticides with water and gives an indication of solvent polarity. Other practical considerations such as cost, safety, and hazardous waste disposal also play a role in the selection of the solvent(s) used in a method. Combinations of solvents permit somewhat more control in extraction.

The polarity index is a measure of the polarity of the solvent, which is often the most important factor in the solvent choice for the particular application. In extraction processes, the tenet that 'like dissolves like' (and conversely, 'opposites do not attract') is the primary consideration in choosing the solvent for extraction, partitioning, and/or analytical conditions. For example, hexane often provides a selective extraction for nonpolar analytes, and toluene may provide more selectivity for aromatic analytes.

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Ideally, no solvent exchanges would be needed in a method, but the final extract is usually not in the same solvent as the initial extract.

For multiclass, multiresidue pesticide methods, acetone, acetonitrile, and ethyl acetate have been shown to give high recoveries for a wide range of pesticides, each with some advantages over the other solvents. The ability to remove water from the initial extract is critical in providing a higher degree of selectivity in high-moisture samples, such as foods. Co-extraction of proteins, sugars, and other polar compounds tends to increase in conjunction with the amount of water in the extract, so solvents that avoid the water give greater selectivity. Ethyl acetate and acetonitrile are better than acetone for this purpose because water can more easily be removed by the use of salting out. Nonpolar co-solvents are needed in the case of acetone, which leads to dilution of the extract and greater co-extraction of lipids. Ethyl acetate also extracts lipids readily, which makes acetonitrile the most advantageous solvent in multiclass, multiresidue methods for pesticides in food analysis.

In situations involving acidic/basic analytes, pH is often the most critical property in the extraction, and buffered aqueous solvents are often necessary. Another important consideration is the stability of the analytes in the extraction medium, and method development should entail analyte stability experiments to demonstrate how long solutions and/or extracts can be stored.

2.1.1 Blending and sonication

Blending or mixing the sample with the solvent is a time-tested and simple approach that is very fast, convenient, and inexpensive. Assuming extraction efficiency is acceptable, simply shaking the sample with solvent should be the first choice in extractions owing to the following advantages: (1) no external device is introduced, thus minimizing the potential for contamination of the sample; (2) vortex mixers or other types of mechanical shakers are cheaper and require less maintenance than blending or other types of extraction devices; (3) batches of samples can be processed simultaneously to increase throughput; (4) shaking of the sample can be done by hand in the field if needed; (5) nothing needs to be cleaned between samples, and potential for carryover is reduced; (6) no solvent is removed or added as in the case of a probe device; and (7) metal surfaces are avoided and mechanical heating does not occur, which lessens the chance of adsorption, reactions, and/or thermal degradation.

The main disadvantage with shaking relates to the potential for lower extraction efficiency of incurred residues, depending on the types of samples and analytes. In the case of fruits and vegetables, however, even field-incurred pesticides can often be extracted easily with solvent at room temperature and pressure conditions. Ideally in method development research (and a requirement for registration of pesticides), the total extractability of residues is determined using radiolabeled pesticides incurred into the samples as they are used in the field. This is a very expensive study, however, and in the absence of these materials, the next best approach is to evaluate extractability using a standard reference material (SRM). Unfortunately, SRMs are available only on a limited basis for certain pesticides and matrices (e.g., organochlorinated pesticides in environmental sample types). An alternative approach is to compare the results from a new extraction method with the results using an established and rigorously validated extraction method for field-incurred samples. Recoveries from fortified samples in

the laboratory are not acceptable to evaluate truly the performance of an extraction method, and studies that only present results from experiments with fortified samples should not be trusted when accurate results are paramount.

In the case of matrices such as clay soils that tightly retain certain analytes, sonication using a high-energy probe is an extraction approach that can often break matrix–analyte interactions. However, owing to the higher energy input involved, sonication has a greater potential than simple blending for degrading analytes, but the approach can be useful for stable analytes.

2.1.2 Microwave-assisted extraction (MAE)

MAE simply involves placing the sample with the solvent in specialized containers and heating the solvent using microwave energy. MAE is also sometimes called MASE, which can stand for microwave-assisted solvent extraction or microwave-accelerated solvent extraction. In any event, the extraction process is more rapid than Soxhlet extractions, can be run in batches, and reduces solvent consumption. As in the case of sonication, MAE may overcome retention of the analyte by the matrix, but analyte degradation can be a problem at higher temperatures in certain applications. The solvent, microwave energy applied, and extraction time selected are the main parameters controlled in MAE. The user should use proper extraction vessels and equipment in MAE because very high pressures can be achieved and explosions may result if appropriate precautions are not taken.

One application using MAE is a method to determine imidazolinone herbicides and their respective metabolites in plant tissue.[4] Current residue methodologies for determining imazethapyr (imidazolinone herbicide) and its metabolites in crops involve laborious, time-consuming cleanup procedures after an aqueous/organic extraction. MAE in conjunction with high-performance liquid chromatography/mass spectrometry (HPLC/MS) shortened the cleanup procedure and provided satisfactory recoveries (97–102%) for the parent imazethapyr and its two metabolites (hydroxy and glucose conjugate metabolites).

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2.1.3 Pressurized liquid extraction (PLE)

PLE is also commonly referred to as accelerated solvent extraction, under the registered trade name ASE or the trade name pressurized solvent extraction (PSE) by another vendor. The instrumental approach generally involves first dispersing the sample with an inert material (e.g., drying agent or sand) and placing the mixed sample in an extraction vessel. The approach consists of introducing the solvent into the vessel followed by heating of the vessel and a static extraction step (no flow). After this ca 5–20-min step, flow is initiated (dynamic extraction step), and the extract is collected in a vial. The process may be repeated if needed to increase analyte recoveries. Although increased temperature is not a necessity in PLE, higher temperature is usually used to speed the extraction and break analyte–matrix interactions.

The order of importance of parameters for an application in PLE (and extraction in general) is typically: (1) solvent; (2) temperature; (3) time; (4) repetitions; and (5) pressure. The same types of solvents can be used in PLE as in traditional approaches, but relatively viscous solvents, such as ethanol and water, can be difficult to flow

through the sample even at high pressures. Also, highly acidic and basic conditions can be damaging to instrument components, which limits the use of PLE in certain applications. The properties of solvents can change dramatically at different temperatures and pressures (the boiling point at room temperature is commonly exceeded in PLE and MAE); hence, if possible, potentially more hazardous solvents should be replaced with more benign solvents. PLE has been validated for US Environmental Protection Agency (EPA) Method 3545A, which entails the extraction of several nonpolar organic contaminants, including many semi-volatile pesticides, from different types of environmental solid samples. Another method using PLE, that was validated (as a tolerance enforcement method) by the US EPA Office of Pesticide Programs (OPP) Laboratory, is a method developed by DuPont Crop Protection (Wilmington, DE, USA).[5] This method called for extracting cotton gin trash, using PLE, followed by liquid–liquid partitioning, and determination by liquid chromatography/tandem mass spectrometry (LC/MS/MS), using atmospheric pressure chemical ionization (APCI).

2.1.4 Supercritical fluid extraction (SFE)

SFE is an instrumental approach not unlike PLE except that a supercritical fluid rather than a liquid is used as the extraction solvent. SFE and PLE employ the same procedures for preparing samples and loading extraction vessels, and the same concepts of static and dynamic extractions are also pertinent. SFE typically requires higher pressure than PLE to maintain supercritical conditions and, for this reason, SFE usually requires a restrictor to control better the flow and pressure of the extraction fluid. CO_2 is by far the most common solvent used in SFE owing to its relatively low critical point (78 atm and 31 °C), extraction properties, availability, gaseous natural state, and safety.

A major advantage of SFE over liquid-based methods is that the extraction solvent becomes a gas after extraction and the analytes are conveniently concentrated in the collecting medium (solid-phase trap or liquid). Liquid methods nearly always require a concentration step after extraction. Another key advantage of SFE is that the density of the supercritical fluid and other physicochemical properties can be dramatically altered through control of temperature and pressure. This permits a higher degree of selectivity and versatility in the extraction process without having to use different solvents. In some cases, SFE can eliminate post-extraction cleanup steps or at least make cleanup using SPE very convenient by using the SPE sorbent as a trapping medium in SFE.

However, SFE also has several disadvantages, which has caused more than one vendor to terminate their SFE instrument product lines. The higher selectivity of SFE limits the range of analytes that can be extracted under the same conditions. Furthermore, SFE can have difficulty in overcoming analyte–matrix interactions in certain applications (soils in particular). Organic solvents (and water), often called 'modifiers' in SFE, are sometimes added to the supercritical fluid to increase the polarity range of the extraction process and to help overcome analyte retention in the matrix. Other problems with SFE include the high cost of automated instruments, relatively small sample sizes, and a more involved method development process. SFE has been demonstrated to be effective in the extraction of a variety of residues from a variety of matrices. For example, a method using SFE for the determination

of multiple pesticides in nonfatty foods has recently achieved First Action Official Method status by AOAC International,[6] but in the foreseeable future, SFE is unlikely to overcome its drawbacks and negative perceptions.

2.2 Cleanup

The separation of analytes from undesirable matrix components, or 'cleanup', of sample extracts can be accomplished through a variety of techniques that take advantage of differences in the physicochemical properties of the analytes from co-extracted matrix components.

2.2.1 Liquid–liquid partitioning

In liquid–liquid partitioning, water does not necessarily have to be one of the solvents for cleanup applications. For example, hexane and other nonpolar solvents can partition with acetonitrile or methanol to remove lipids but leave relatively polar pesticides in the other organic solvent phase. Common disadvantages with liquid–liquid partitioning include the typical use of large volumes of potentially hazardous solvents, necessity for subsequent concentration steps, generation of hazardous waste, labor-intensive and/or time-consuming procedures, potential occurrence of emulsions, the need for laboratory hood and storage space, added external source of potential sample contamination, and the dishwashing requirements of the glassware. The main advantages of the approach are its effectiveness, low cost, and ease of use. The use of pH variations is often critical and can help either to separate ionic pesticides from neutral compounds or to separate neutral pesticides from ionic compounds.

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2.2.2 Gel permeation chromatography (GPC)

GPC separates molecules by size and thus achieves minimal losses of pesticides when separating fats and other large molecules by taking advantage of the molecular size differences of the larger chemicals from the smaller pesticides. Because many pesticides tend to have relatively similar molecular size, they can generally be collected in a single fraction. Another advantage is that the GPC column can be re-used many times and is easily automated, unlike typical uses of adsorbent columns. Unfortunately, many matrix co-extractives of similar molecular size as the pesticides are not separated in GPC, and other cleanup techniques may still be required afterwards. Practical disadvantages of GPC include the very large volumes of potentially hazardous solvents involved, generation of waste, the need for post-GPC concentration steps, and the costs associated with purchase and maintenance of instrumentation and columns. In recent years, new gel materials, more benign solvent combinations, and miniaturized columns have improved practical aspects of GPC, and this technique remains the most common means to remove high, and very low, molecular weight components from food and environmental samples. In the case of high-fat matrices (>25%), GPC is nearly always used to remove the lipids prior to analysis because alternative methods such as co-sweep distillation are not as practical or effective.

2.2.3 Solid-phase extraction (SPE)

The most common and diverse approach to cleanup (and extraction of water samples) in pesticide residue analysis is SPE. Over the last 20 years, improvements and diversifications in SPE formats, sorbent types, and apparatus have made SPE a widely used approach for a variety of applications, including the analysis of pesticide residues. SPE cartridges or disks can be likened to low-resolution HPLC columns in that similar stationary and mobile phases are used. A typical particle size in SPE is 40 μm, and the plastic cartridges are generally packed with 0.1–1 g of sorbent in plastic tubes. The choice of reversed-phase, normal-phase, and ion-exchange media in SPE is very diverse, and Table 2 lists some of the more popular SPE applications for the cleanup of pesticides.

Reversed-phase (C_1–C_{18}) sorbents retain many types of organic species from water, but organic solvents generally cause them to elute easily from the sorbent, which is why C_{18} and similar sorbents are most commonly used for concentrating analytes from water. In cleanup applications, C_{18} can be used to rinse salts and highly polar organics from a solution and perhaps retain the most highly nonpolar compounds in an extract. However, the most polar pesticides may start to break through the sorbent bed when larger water volumes are used, which acts to decrease recoveries and/or increase detection limits in analytical water methods. In combination with pH adjustments, C_{18} and similar types of reversed-phase sorbents can be useful in separating acid/base pesticides.

The use of polymer-based sorbents has begun to replace the traditional silica-based reversed-phase sorbents. Divinylbenzene–styrene and proprietary polymers for SPE have proliferated in the market and will likely replace most C_{18} applications in the future. The polymer sorbents give stronger retention, have greater capacity per unit weight/volume, do not need preconditioning, can be allowed to go dry during and after use, and have greater permeability (and thus work faster and do not clog as easily).

Sorbents such as aminopropyl ($-NH_2$) and primary secondary amine (PSA) strongly retain polar organics such as sugars, humic acids, and fatty acids, which are common in food and environmental samples. Graphitized carbon black (GCB) is noteworthy for strongly retaining all types of planar molecules, such as color-producing components and sterols. These two sorbents, singly or in combination, can be very useful for

Table 2 Common SPE sorbents used in analytical methods for pesticides

Normal-phase	Reversed-phase	Ion-exchange
Graphitized carbon black (GCB)	Octadecylsilane (C_{18})	Strong anion-exchange (SAX)
Alumina (acid, base, neutral)		Primary secondary amine (PSA)
Silica	Polymer (DVB,[a] other)	Aminopropyl ($-NH_2$)
Florisil	Cyanopropyl ($-CN$)	Strong cation-exchange (SCX)

[a]DVB = divinylbenzene.

cleaning up complicated extracts containing pesticide residues, provided that the pesticides are not too polar or planar. These sorbents are so retentive that if the pesticides are adsorbed, the retention may be irreversible. Thus, the most effective usage of these sorbents involves 'chemical filtration' of matrix components rather than the 'retention–elution' approach of the analytes as used for water analysis and other common SPE applications.

A very simple, effective, and inexpensive SPE approach that can be used when the interfering matrix components are retained by the sorbent but pesticides remain in the liquid mobile phase (i.e., chemical filtration applications) is 'dispersive SPE'.[7] Dispersive SPE involves the mixing of a small amount of the SPE sorbent with the extract in a vial rather than in a column format. Centrifugation, gravity settling, or filtration can be used to separate an aliquot of the extract from the sorbent after dispersive SPE. This approach has several advantages over the traditional cartridge format, which include: (1) all of the sorbent has equal access to the extract, and thus the cleanup is more effective and efficient; (2) less sorbent and no cartridges are needed, and thus costs are greatly reduced; (3) no SPE manifold, collection tube, vacuum apparatus, or other extraneous supplies are needed as with traditional SPE formats; (4) no elution solvent is needed, thus no dilution of the extract occurs, and a post-SPE solvent evaporation step may be avoided; (5) no pretreatment of the sorbent is required and no flow occurs, and thus flow control, channeling, and dry columns are no longer concerns; and (6) the procedure is very fast and easy, which saves time and analyst training. If chemical filtration is the type of cleanup that is being conducted, dispersive SPE should be the first choice in the procedure.

SPE using pipet tips is even more convenient than dispersive SPE because a single liquid transfer is all that is involved (for chemical filtration), but only a very small amount of sorbent (e.g., 4 mg) is used, and the cost is fairly high. Otherwise, SPE formats can be cartridges or membrane extraction disks of various sizes designed for single or bi-directional flow. SPE has been commercialized in automated 96-well plate applications to increase sample throughput. Centrifugation can be used instead of suction in another type of format using columns.

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The choice of sorbent–solvent combination in SPE for cleanup of extracts is much like choosing the extraction solvent for certain pesticide(s) in a particular matrix. Experience with SPE and knowledge about the chromatographic behavior of the analytes and matrix co-extractives can save much effort in method development using SPE. However, trial-and-error is the more common approach to method development using SPE because unpredictable results and/or subtle differences may occur between different stationary–mobile phase combinations. In multiclass, multiresidue applications, the need to maintain a wide polarity range of analytes does not allow the analyst to achieve the best degree of selectivity for the analyte as possible in SPE, but SPE conditions can be set fairly selectively for individual analyte types in single analyte or single class methods.

Prior to the development of modern SPE formats, liquid–solid partitioning with charcoal, silica, Florisil, and/or alumina was common to aid in the removal of lipids in the determination of nonpolar pesticides, but these sorbents are less useful in the cleanup of semi-polar and polar pesticides owing to the large elution volumes needed. Applications of modern SPE are discussed in Section 3.2.

3 Analytical separations and detection

For detection, MS is rapidly becoming the method of choice for multiclass, multiresidue analysis owing to its many advantages, recent improvements in technology, and availability of cost-effective commercial instrumentation. Detection systems in general are continually being improved, and in combination with the improvements in chromatographic instruments and techniques, an exceptionally low limit of detection (LOD) is possible for pesticide residues.

3.1 Gas chromatography/mass spectrometry (GC/MS)

The most widely regarded approach to accomplish the determination of as many pesticides as possible in as few steps as possible is to use MS detection. MS is considered a universally selective detection method because MS detects all compounds independently of elemental composition and further separates the signal into mass spectral scans to provide a high degree of selectivity. Unlike GC with selective detectors, or even atomic emission detection (AED), GC/MS may provide acceptable confirmation of the identity of analytes without the need for further information. This reduces the need to re-inject a sample into a separate GC system (usually GC/MS) for pesticide confirmation. Through the use of selected ion monitoring (SIM), efficient ion-trap or quadrupole devices, and/or tandem mass spectrometry (MS/MS), modern GC/MS instruments provide LODs similar to or lower than those of selective detectors, depending on the analytes, methods, and detectors.

MS detection does not necessarily require as highly resolved GC separations as in the case of selective detectors because the likelihood of an overlapping mass spectral peak among pesticides with the same retention time is less than the likelihood of an overlapping peak from the same element. Unfortunately, this advantage cannot always be optimized because SIM and current gas chromatography/tandem mass spectrometry (GC/MS/MS) methods, it is difficult to devise sequential SIM or MS/MS retention time windows to achieve fast GC separations for approximately >50 analytes in a single method.

In 1994, only 15% of EPA method validations (tolerance method validation and environmental chemistry method validations) that involved GC were carried out using GC/MS. In 2002, this number is reversed in that 85% of the GC methods that were validated by both programs used GC/MS. Many of the compounds investigated in these method trials were polar compounds, and hence these compounds required derivatization in order to be amenable to GC. One common methylating agent is (trimethylsilyl)diazomethane, which is used, for example, to methylate the sulfonamide flumetsulam. As opposed to HPLC/MS, where derivatization is often not necessary, the GC/MS procedure involves an extra step to methylate this compound, under dry conditions, prior to determination by GC/MS.

Another GC/MS method that was validated as a food tolerance method involved the determination of glyphosate and (aminomethyl)phosphonic acid (AMPA) in crops.[8] In this method, glyphosate and AMPA residues are extracted from crop commodities (corn grain) with water. The extracts are then partitioned with dichloromethane,

and the aqueous layer is subjected to cation-exchange SPE cleanup. The analytes in the purified extracts are derivatized directly using a mixture of trifluoroacetic anhydride and heptafluorobutanol. The amine functional groups are derivatized to form the corresponding trifluoroacetyl derivatives. The carboxylic and phosphonic acid functional groups are derivatized to form the corresponding heptafluorobutyl esters. After derivatization, the excess reagents are evaporated, and the residue is dissolved in ethyl acetate and analyzed by GC/MS. The limit of quantitation (LOQ) for both compounds in corn grain is 0.05 mg kg^{-1}, and this approach has also achieved AOAC International Official Method status.[8]

3.1.1 Fast GC/MS

Increasing the speed of analysis has always been an important goal for GC separations. All other parameters being equal, the time of GC separations can be decreased in a number of ways: (1) shorten the column; (2) increase the carrier gas flow rate; (3) reduce the column film thickness; (4) reduce the carrier gas viscosity; (5) increase the column diameter; and/or (6) heat the column more quickly. The trade-off for increased speed, however, is reduced sample capacity, higher detection limits, and/or worse separation efficiency.

In practice, the GC conditions should be designed to give the shortest analysis time while still providing the necessary selectivity (i.e., separation of both analyt–analyte and matrix–analyte). Selective detectors often have fast data collection rates and improved matrix–analyte selectivity, but analyte–analyte selectivity must be addressed solely by the GC separation. MS can improve both types of selectivity and, by reducing the reliance on the GC separation, faster analysis times can often be achieved in complicated mixtures.

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The full-scan mode is needed to achieve completely the full potential of fast GC/MS. Software programs, such as the automated mass deconvolution and identification system (AMDIS), have been developed to utilize the orthogonal nature of GC and MS separations to provide automatically chromatographic peaks with background-subtracted mass spectra despite an incomplete separation of a complex mixture.[9] Such programs in combination with fast MS data acquisition rates have led to very fast GC/MS analyses.

There are at least three approaches to fast GC/MS: (1) use of microbore columns with time-of-flight mass spectrometry (TOFMS);[10] (2) use of low-pressure (LP)-GC/MS to aid separations at increased flow rate;[11] and (3) use of supersonic molecular beam mass spectrometry (SMBMS) (also known as supersonic GC/MS), which can accept increased flow rates and short analytical columns.[12]

An advantage of the microbore gas chromatrography/time-of-flight mass spectrometry (GC/TOFMS) method over the other two approaches is that separation efficiency need not be compromised for speed of analysis. The rapid deconvolution of spectra ('scan rate') with TOFMS makes it the only MS approach to achieve several data points across a narrow peak in full-scan operation. However, the injection of complex extracts deteriorates performance of microbore columns quickly, and an increased LOD and decreased ruggedness result. Microbore columns may be used in water analysis if the LOD is sufficiently low, but they can rarely be used in real-life applications to complicated extracts.

LP-GC/MS, commercially known as rapid MS, is an interesting approach to speed the analysis by which a relatively short (10-m) megabore (0.53-mm i.d.) column is used as the analytical column. The vacuum from the mass spectrometer extends into the column, which leads to higher flow rates and unique separation properties. A restriction capillary (0.1–0.25-mm i.d. and appropriate length) is placed at the inlet end to provide positive inlet pressure and allow normal GC injection methods. Advantages of LP-GC/MS include the following: (1) fast separations are achieved; (2) no alterations to current instruments are needed; (3) sample capacities and injection volumes are increased with megabore columns; (4) peak widths are similar to those in conventional separations to permit normal detection methods; (5) peak heights are increased, and the LOD can be lower (depending on matrix interferences); (6) peak shapes of relatively polar analytes are improved; and (7) thermal degradation of thermally labile analytes is reduced. However, reduced separation efficiency occurs with LP-GC/MS, and the ruggedness of the approach with repeated injections was no better than that of traditional methods with a narrow-bore analytical column.

GC/MS with current commercial instruments has a practical flow limitation of $2\,\text{mL}\,\text{min}^{-1}$ due to MS instrument designs. Gas chromatography/supersonic molecular beam mass spectrometry (GC/SMBMS) is a very promising technique to overcome this flow rate limitation because SMBMS requires a high gas flow rate at the supersonic molecular beam (SMB) interface. However, only a single prototype GC/SMBMS instrument exists at present, and the approach is not commercially available. The advantages of GC/SMBMS include the following: (1) the selectivity of the MS detection with electron ionization is increased because an enhanced molecular ion occurs for most molecules at the low temperatures of SMB, so losses of selectivity in the GC separation can be made up for by increased selectivity in the MS detection; (2) the use of very high gas flow rates allows GC analysis of both thermally labile and nonvolatile chemicals, thereby extending the scope of the GC/SMBMS approach to many analytes currently determined by HPLC; (3) the SMBMS approach is compatible with any column dimensions and injection technique; (4) reduced column bleed and matrix interference occur owing to lower temperatures and enhanced molecular ions; and (5) better peak shapes occur because tailing effects in MS are eliminated.

3.1.2 Pesticide identification

Confirmation criteria in pesticide residue analysis are a subject of debate, depending on the application, but human judgment is required in the final decision. The use of two different columns in chromatography in the absence of MS confirmation is becoming less satisfactory in the view of many pesticide analytical chemists. For MS, typical confirmation criteria include: (1) proper chromatographic retention time; (2) three ions of correct mass/charge (m/z) ratios; (3) adequate signal-to-noise (S/N) ratios; and (4) absence of similar signals in blank samples (to indicate no contamination or carry-over). The definitions of 'proper', 'correct', and 'adequate' are based on the need and importance of the result. In any case, some pesticides do not provide three ions in their spectra, or the ion ratios for some spectra may be more variable than deemed acceptable if strict definitions are used. Other factors are sometimes taken into account, such as chromatographic peak shape, sample history, and/or analyst experience. In these difficult cases, information from other analyses, perhaps using other

ionization modes in MS or other detection systems, is sometimes used to provide more evidence.

The combination of an element-selective detector and MS can provide excellent information for determining the presence of an analyte or, just as important, eliminating the chance that a pesticide is present. The added selectivity gained in the use of tandem MS (MS^n) can also weigh heavily in confirmation of pesticide identity despite the possible lack of three ions of defined ratios.

Another concept gaining prominence in the community is the use of 'identification points', in which a confirmation is made when a certain number of assigned points are collected in an analysis.[13] For example, each ion in standard MS counts for 1 point, and 1.5 points may be given for MS–MS product ions, or 2 points may be assigned for ions obtained with enough MS resuloution to achieve accurate mass determinations. According to this system, when 3–4 points are accumulated (depending on the application), then a confirmation is said to be made. However, human judgment should still play a role because the point assignments are arbitrary and actual rates of false positives and negatives are typically unknown. It makes sense that the more points that are gathered in an analysis should lead to a confirmation, but MS should not be considered as the only tool in the analysis, and all the pieces of information should make sense and point in the same direction for most accurate confirmations.

3.2 HPLC/MS

Coupling HPLC to a mass spectrometer is far more complicated than in a GC system because of the large amount of mobile phase solvent expanding into the system (see Table 1 for expansion volumes). Typical mobile phase flow rates for HPLC are 0.5–2 mL min^{-1}, which translates into gas flow rates of 100–3000 mL min^{-1}.

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During the past 20 years, researchers have used several approaches to remove the solvent during ionization. Among the different interfaces, thermospray ionization was used because of its compatibility with HPLC system mobile phases, but thermospray has its limitations such as imprecision, compound-dependent responses, and limited ruggedness.[14] The particle beam interface[15,16] has been commonly used to generate electron ionization (EI) spectra in HPLC/MS. However, the importance of the particle beam is declining, mainly because of insufficient sensitivity. Table 3 shows how HPLC/MS technology has evolved in the past 25 years. Commercial implementation of the approaches is several years behind in the initial invention of the different approaches, and only in the mid-1990s did instrumentation achieve high-quality results

Table 3 Evolution of LC/MS

Year	Interface
1974	Moving wire/belt
1978	Direct liquid introduction
1980	Thermospray
1984	Particle beam
1985	Continuous fast atom bombardment
1985	Electrospray/APCI

and sufficiently low detection limits for most pesticide applications using atmospheric pressure ionization (API) in HPLC/MS.

Full acceptance of HPLC/MS methods by the US EPA OPP as enforcement methods occurred between 1998 and 2001. For example, in 1998, the EPA OPP accepted HPLC/MS (without MS/MS) methods as primary enforcement methods, and high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) only was suitable for confirmatory methods. However, in 2001, HPLC/MS/MS methods also became acceptable for primary enforcement. Table 4 summarizes the types of methods that were validated by the EPA OPP method validation program, for both food tolerance enforcement methods and environmental chemistry methods.

Electrospray ionization (ESI) and APCI are the two popular API techniques that will be discussed here. The applications to the analysis of pesticides that will be discussed include imidazolinone herbicides, phenoxy acid herbicides, and *N*-methyl carbamate insecticides. Matrix effects with respect to quantitation also will be discussed. For the

Table 4 Overview of LC/MS methods submitted to the EPA OPP

Chemical	Matrix	Ionization mode	MS or MS/MS	LOQ ($\mu g\,kg^{-1}$)
Cymoxanil	Soil	ESI	MS	50
Cymoxanil	Water	ESI	MS	2.0
Fosamine ammonium	Soil	ESI	MS	50
Imazapic	Peanuts	Thermospray	MS	NA[a]
Imazethapyr	Alfalfa hay	Thermospray	MS	NA[a]
Iprodione	Soil	ESI	MS/MS	10
Iprodione	Water	ESI	MS/MS	0.05
Isoxaflutole	Soil	ESI	MS/MS	0.40
Isoxaflutole	Water	ESI	MS/MS	0.01
Kresoxim-methyl	Soil	ESI	MS/MS	0.50
Kresoxim-methyl	Water	ESI	MS	0.05
Mefenoxam	Canola seed	ESI	MS	10
Mefenoxam	Dried basil	ESI	MS	10
Mefenoxam	Fresh mint	ESI	MS	10
Mefenoxam	Star fruit	ESI	MS	10
Methoxyfenozide	Apple	ESI	MS	10
Methoxyfenozide	Cottonseed	ESI	MS	10
Methoxyfenozide	Cotton gin trash	ESI	MS	10
Methoxyfenozide	Cottonseed oil	ESI	MS	10
Methoxyfenozide	Milk	ESI	MS	10
Methoxyfenozide	Beef liver	ESI	MS	10
Primisulfuron-methyl	Soil	ESI	MS	0.50
Primisulfuron-methyl	Water	ESI	MS/MS	0.05
Prometryn	Soil	ESI	MS/MS	10
Prosulfuron	Soil	ESI	MS/MS	0.50
Prosulfuron	Water	ESI	MS/MS	0.05
Pyrithiobac sodium	Cotton gin trash	APCI	MS/MS	20
Quinclorac	Soil	ESI	MS	10
Tebufenozide	Wheat straw	ESI	MS	20
Thiamethoxam	Cottonseed oil	ESI	MS/MS	10
Tralkoxydim	Soil	ESI	MS/MS	10

[a]Confirmatory method for a capillary electrophoresis/UV method.

discussions concerning the ESI and APCI techniques, references 17–26 were used, and these references are highly recommended, particularly for individuals just getting started with atmospheric pressure ionization mass spectrometry (API-MS).

3.2.1 ESI

In an electrospray ion source, the mobile phase is forced through a narrow metal capillary needle at a high electrical potential relative to the walls of the atmospheric pressure region. The potential causes the mobile phase to explode into a fine spray of charged droplets; a drying gas helps the solvent evaporate rapidly. The dry solute ions formed are transferred from the atmospheric pressure region into the mass analyzer through a low-pressure transport region [$\sim$1 Torr = (1 Torr = 133.3 Pa)]. The transport region is equipped with a heated capillary, a skimmer, and lens arrangements. The electrospray interface uses an additional octapole lens for ion focusing and 'in-source' collision-induced dissociation (CID) experiments (in the case of a single-quadrupole instrument).

The ability to form analyte ions from the electrospray process depends on the pH and mobile phase composition. To generate analyte ions from the mobile phase, a volatile organic acid such as formic acid or acetic acid is added to the mobile phase. The addition of acids favors the generation of positive ions, $[M + H]^+$, but is not the first choice for negative ions, $[M - H]^-$. The ESI process is 'soft', generating $[M + H]^+$ or $[M - H]^-$ ions even for very thermally unstable and nonvolatile molecules. The choice of mobile phase is very important in that it must be volatile and not have strong ion pairing properties. For example, HPLC mobile phases containing acetic acid, formic acid, and ammonium acetate are acceptable. However, HPLC mobile phases containing nonvolatile buffers, such as phosphate buffers, are not acceptable because their strong ion pairing properties will favor the formation of neutral products. Stronger acids such as trifluoroacetic acid (TFA), although frequently used with HPLC, are less desirable because strong acid anions pair with analyte cations, thus reducing the analyte ion abundance. Ion pairing relative strengths are sulfates, borates, phosphate > TFA > formate > acetate. Table 5 lists what solvents are acceptable for both ESI and APCI.

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The applicable HPLC flow rate with ESI is lower than that with thermospray or APCI, usually below the 0.5 mL min^{-1} range. The typical flow rate is 0.10–0.20 mL min^{-1} for ESI, which means that the effluent flow introduced into the electrospray must be reduced by splitting when using a conventional HPLC column (4.6-mm i.d. $\times$ 250 mm). Currently, narrower columns (e.g., 2.1-mm i.d.) and slower flow rates are commonly used to achieve the desirable flow rates. The advantage of this approach is that improved separation efficiency and faster separations are also achieved (at the cost of sample capacity).

ESI performs well for the more polar compounds such as imidazolinone herbicides, sulfonylurea herbicides, triazine herbicides, phenoxy acid herbicides, and carbamate pesticides (to name a few). ESI also performs well with proteins and peptides.

3.2.2 APCI

The APCI interface uses pneumatic nebulization in an atmospheric pressure region to form fine spray droplets. Typically, these systems use a heated nebulizer (300–650 °C)

Table 5 Solvents and additives compatible for API-MS

Suitable for ESI and APCI	Not suitable for ESI
Methanol	Toluene
Ethanol	Benzene
Propanol	Hydrocarbons (e.g. hexane)
2-Propanol	Styrene
Butanol	Carbon tetrachloride
Acetonitrile	Carbon disulfide
Water	
DMF (10% or less)	
DMSO (10% or less)	
Acetic acid	
Formic acid	
Ammonium acetate	
Ammonium hydroxide	
Trifluoroacetic acid (TFA)	
Triethylamine (TEA)	

for spray formation at atmospheric pressure, and subsequently chemical ionization is achieved by a corona discharge in the same region. The probe is connected to the mass analyzer through a transport region that is identical with that used in an electrospray system. In fact, modern API systems allow a rapid change between the ESI and APCI modes using two different API modules. The disadvantage is that the operator cannot simultaneously operate in the two modes (APCI and ESI). Table 5 shows the appropriate solvents and additives that can be used in APCI.

As in the case of thermospray techniques, the analyte response usually depends on the proton affinities of the analytes in APCI. Compounds with high proton affinities usually show high analyte responses. Therefore, APCI is often more selective than ESI, and APCI is generally more useful for less polar compounds analyzed using HPLC, such as alcohols, aldehydes, ketones, and esters. Because vaporization temperatures in APCI are between 300 and 600 °C, thermally labile compounds will not perform as well in APCI as in ESI. Certain compounds in the mid-polarity range, such as carbamate pesticides, perform well with both APCI and ESI. In this situation, ESI will usually give better sensitivity, but the design of the API source also makes a big difference in the efficiency of ions entering the mass analyzer and detection limits with ESI and APCI.

There are several advantages and disadvantages in using each API technique. For example, the US Geological Survey has made extensive comparisons between APCI and ESI for 30–75 pesticides.[27,28] Neutral pesticides in solution are often more sensitive in APCI (especially positive ion), and ionic pesticides in solution are more sensitive in ESI. Also in ESI, the formation of sodium adducts is fairly common, but not in APCI. Another advantage of APCI is that higher HPLC flow rates (0.5–2 mL min^{-1}) are possible, which permits a greater sample capacity. Also, matrix effects (signal suppression or enhancement) are common with ESI but occur to a lesser extent with APCI (examples later will demonstrate how APCI is less susceptible to matrix effects than is ESI).

3.2.3 Optimization of API-MS response

Figure 1 shows a flow chart that illustrates the recommended steps needed for optimizing the API signal and HPLC/MS method. If the API signal is not satisfactory, performance can be greatly improved with post-column modification of the LC solvent. This is achieved by adding a post-column mixing tee and a pump (capable of delivering 4–400 μL min^{-1} of modifier). For example, 2-propanol can be added at ~0.10 mL min^{-1} to aid in the desolvation of aqueous solvents and dilute ionic buffers, in order to achieve acceptable API-MS performance. Also sodium acetate (50 μM) can be added post-column to aid in the cationization of samples, particularly for samples that have weak sites for protonation. If TFA must be used in the mobile phase, the ESI sensitivity can be improved by adding 20% propionic acid–80% 2-propanol (also known as the 'TFA fix'). In this case, the propionic acid displaces the TFA based on volatility, which favors the formation of $[M + H]^+$.

Triethylamine (TEA) is sometimes used as an additive for signal enhancement. However, in the positive ESI mode, TEA readily ionizes to give an intense $[M + H]^+$ ion at m/z 102. This then suppresses the ionization of the less basic compounds in the positive ESI mode. In the negative mode, TEA can enhance ionization for certain compounds because of its basic properties.

Other examples, using post-column modification, will be described in Section 3.2.5.

3.2.4 Imidazolinone herbicides

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The utility of high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) and high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) for method simplification in pesticide residue analysis has been well demonstrated recently for the determination of imidazolinone herbicides and their metabolites (Figure 2) in a variety of matrices.[29] With the sensitivity and specificity of HPLC/ESI-MS, the parent compounds (structures A–F in Figure 2) were directly determined in water at 1 ng mL^{-1}.[4] In comparison with the hundreds of milliliters of water, several cartridges, and organic solvents used in conventional procedures,[30,31] only a simple filtration was required prior to HPLC/ESI-MS analysis. The sample throughput was ca 6 samples per day by the conventional methods, but that for HPLC/ESI-MS was only limited by how fast water could be forced through a 0.22-μm filter from a 10-mL disposable syringe.

For the determination of imazethapyr and its metabolites (Structures E, G, and H in Figure 2) in a variety of plant commodities,[32] the amount of initial extract requiring processing was reduced at least 20-fold to 100 mg equivalents from the 2–4 g equivalents typically processed by conventional procedures.[30,33] With the reduced sample requirements and the specificity of HPLC/ESI-MS, the initial extract could be directly loaded on to a 500-mg strong cation-exchange (SCX) cartridge for cleanup eliminating two evaporation steps, a precipitation step, and a C_{18} cartridge from the conventional route. The sample throughput in this case with HPLC/MS increased about 4-fold over the traditional methods. More importantly, this same generic approach using HPLC/MS was successfully demonstrated on each of 11 commodities in

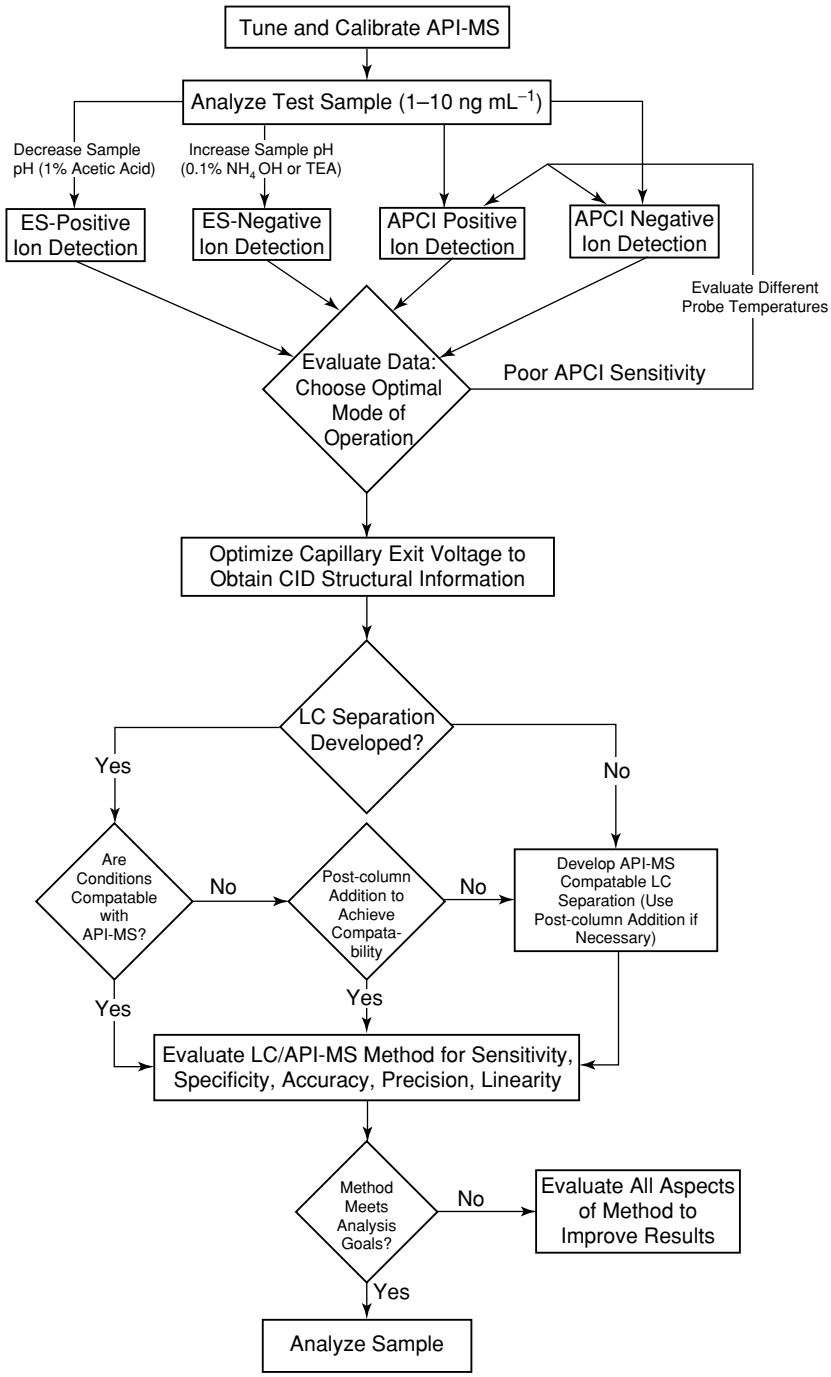
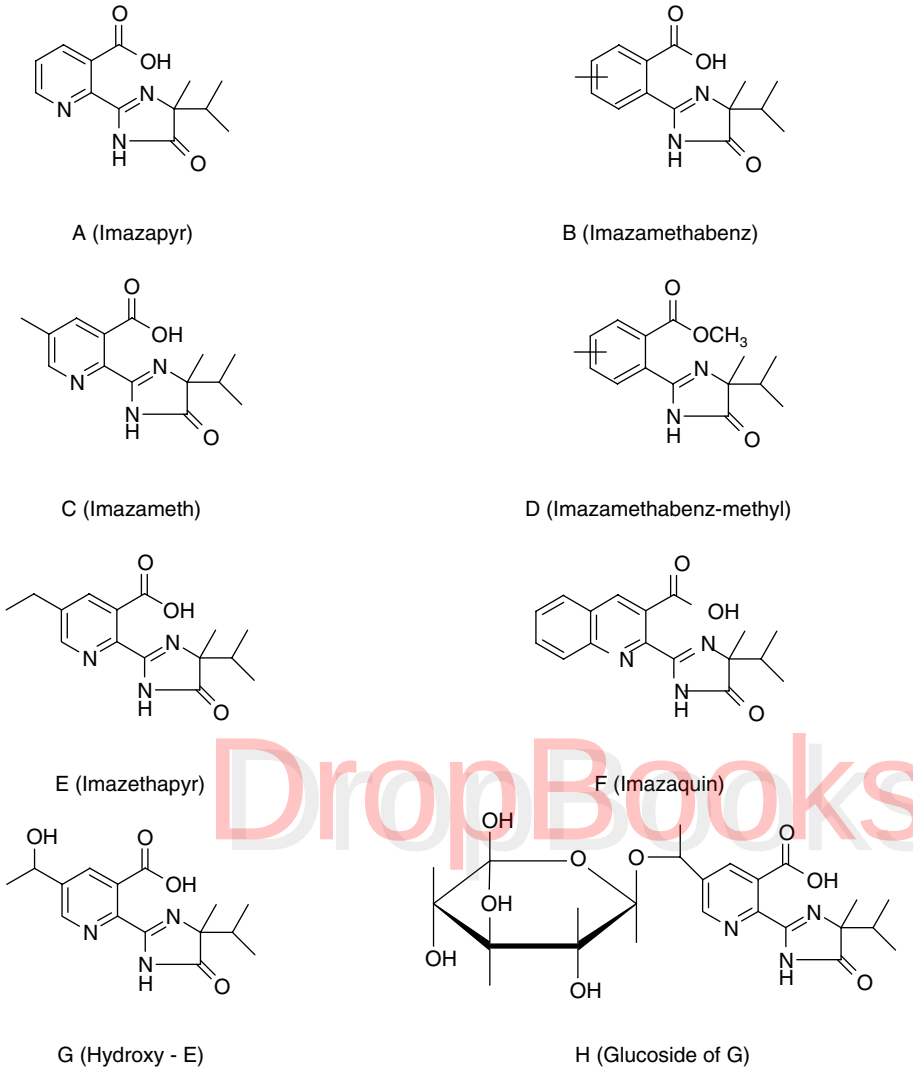

Tune and Calibrate API-MS
Analyze Test Sample (1–10 ng mL^{-1})
Decrease Sample pH (1% Acetic Acid)
Increase Sample pH (0.1% NH_4OH or TEA)
ES-Positive Ion Detection
ES-Negative Ion Detection
APCI Positive Ion Detection
APCI Negative Ion Detection
Evaluate Different Probe Temperatures
Evaluate Data: Choose Optimal Mode of Operation
Poor APCI Sensitivity
Optimize Capillary Exit Voltage to Obtain CID Structural Information
LC Separation Developed?
Yes
No
Are Conditions Compatable with API-MS?
No
Post-column Addition to Achieve Compata-bility
No
Develop API-MS Compatable LC Separation (Use Post-column Addition if Necessary)
Yes
Yes
Evaluate LC/API-MS Method for Sensitivity, Specificity, Accuracy, Precision, Linearity
Method Meets Analysis Goals?
No
Evaluate All Aspects of Method to Improve Results
Yes
Analyze Sample

Figure 1 Optimization scheme for API-MS



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Figure 2 Structures of the imidazolinone herbicides

1–2 days. The effectiveness and efficiency of the HPLC/MS approach provide strong time- and labor-saving benefits despite the high initial cost.

In another example, a multiresidue method using HPLC/ESI-MS was developed to determine six imidazolinone herbicides in five different soil types.[34] Good recoveries (80–120%) and adequate sensitivity at the 2.0 ng g^{-1} level were obtained for the compounds investigated. In the method, a 50-g soil sample was extracted for 1 h in 0.5 N NaOH solution. A portion of the extract was acidified, to precipitate the humic acids, and the supernatant was then loaded on to a preconditioned trifunctional C_{18} SPE cartridge and eluted with ethyl acetate. Further cleanup was achieved using a tandem strong anion-exchange (SAX)–SCX SPE combination. Analytes were eluted

Table 6 List of ions monitored for imidazolinone herbicides using 'in-source' CID (HPLC/ESI-MS)

Analyte	Ions: $[M + H]^+$, and fragment ions (relative intensities), m/z
Imazapyr	262 (100), 217 (28), 243 (30)
Imazamethabenz acid (*m*, *p*)	275 (100), 229 (28), 257 (15)
Imazamox	306 (100), 278 (18), 193 (11)
Imazapic	276 (100), 248 (26), 163 (18)
Imazethapyr	290 (100), 177 (34), 230 (19)
Imazaquin	312 (100), 252 (23), 199 (48)

from the SCX cartridge with saturated KCl-methanol. After cleanup, the sample was desalted using an RP-102 SPE cartridge. The sample was diluted to the appropriate volume with water prior to HPLC/MS analysis.

For analysis, the HPLC mobile phase gradient started at 3 : 17 acetonitrile–0.15% acetic acid in water and ended at 9 : 1 acetonitrile–0.15% acetic acid in water (0–32 min). The HPLC column was a Zorbax RX-C8, 2.1-mm i.d. × 150 mm, 5-μm particle size column with a flow rate of 0.2 mL min^{-1} and a 100-μL injection volume.

Quantitation was achieved using a time-scheduled SIM program (positive mode), monitoring the $[M + H]^+$ ions for each compound. Also, the characteristic fragment ions were monitored using 'in-source' CID. A warning that cannot be overemphasized when using 'in-source' CID, unlike MS/MS, is that the extract from the sample must be clean enough to allow the relative abundances from the fragment ions to be matched to a standard (i.e., fragment ions from the matrix could interfere with ions of the same m/z from the analytes, which could affect the ion ratio in comparison with a reference standard). In this experiment, the extracts were clean enough to confirm, successfully, the presence of the residues found in a 2.0 ng g^{-1} fortification of all six imidazolinones investigated. Table 6 lists the ions monitored for confirmation using 'in-source' CID in this method. The confirmation criteria used involved the appropriate retention time (±2%), fragment ions of S/N > 5, and the appropriate ion ratio (±20%) when compared with a standard. A blank must also not produce a positive result.

The utility of HPLC/ESI-MS/MS for method simplification was also illustrated in the determination of imazethapyr in soil at 1 ng g^{-1}.[35] Compared with the laborious conventional approach of processing 20–25 g equivalents of a 0.5 N NaOH extract through an extensive cleanup as shown above[34] with a sample throughput of 2 h per sample,[36] the HPLC/ESI-MS/MS approach required processing of only 200 mg equivalents of soil extract through a single 200-mg C_{18} cartridge. As shown in Figure 3, HPLC/ESI-MS monitoring of the $[M + H]^+$ ion of imazethapyr was almost adequate by itself as a method. For the imidazolinones, CID of the $[M + H]^+$ ion in MS/MS generated principal product ions at m/z 86 and 69, as shown in Figure 4. The additional specificity of monitoring the ion transition of m/z 290 to 86 in MS/MS gave a chromatogram free of interferences (Figure 5). Using HPLC/ESI-MS/MS for detection reduced the sample preparation time to about 10 min per sample.

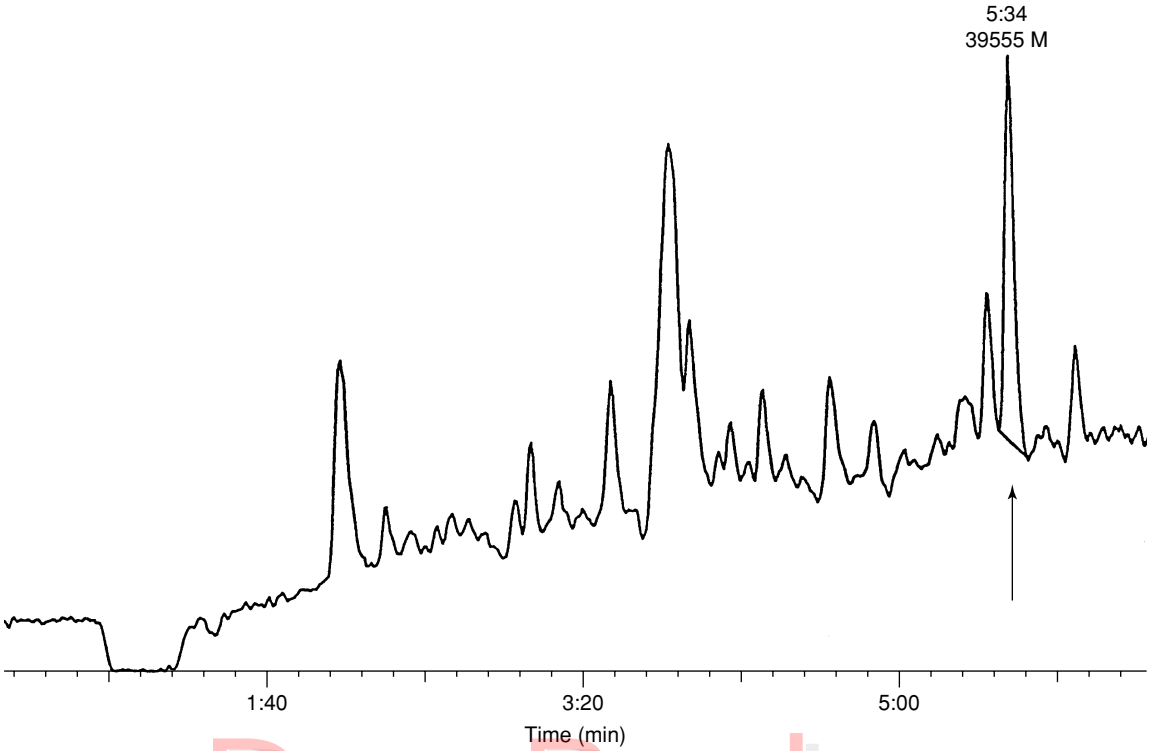


Figure 3 Determination of 1 ng g^{-1} of imazethapyr in soil: HPLC/ESI-MS with SIM of the $[M + H]^+$ ion at *m/z* 290

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3.2.5 Determination of acidic herbicides

Chiron *et al.*[37] used HPLC/ESI-MS in the negative mode for the determination of acidic herbicides in environmental waters. The acidic herbicides investigated were benazolin, bentazone, 2,4-dichlorophenoxyacetic acid (2,4-D), 4-chloro-2-methylphenoxyacetic acid (MCPA), 2-(4-chloro-2-methylphenoxy)propionic acid (MCPP), 4-(4-chloro-2-methylphenoxy)butyric acid (MCPB), and 6- and 8-hydroxybentazone. This method was combined with a prior automated on-line liquid–solid-phase extraction step using an OSP-2 autosampler containing C_{18} cartridges and was applied to the trace determination of acidic herbicides in environmental waters. The proposed method required only 50 mL of water with an LOD between 0.01 and 0.03 ng mL^{-1}, employing SIM of the $[M - H]^-$ ion. Gradient elution was accomplished by changing the eluent from 20% solvent A (methanol) and 80% solvent B (water, pH 2.9 adjusted with formic acid) to 80% A–20% B in 30 min at a flow rate of 0.25 mL min^{-1}. The analysis involved a LiChrospher cartridge column (125 × 3-mm i.d.) packed with LiChrospher 60RP select B material of 5-μm particle size. Post-column addition of 0.1 mL min^{-1} of tripropylamine (4 g L^{-1} methanol) was needed for better sensitivity. Table 7 lists the ions monitored for the above study.

Similar work (unpublished work at the US EPA) was performed for the direct determination of 2,4-D in runoff water at the 1.0 ng mL^{-1} level. Sample preparation

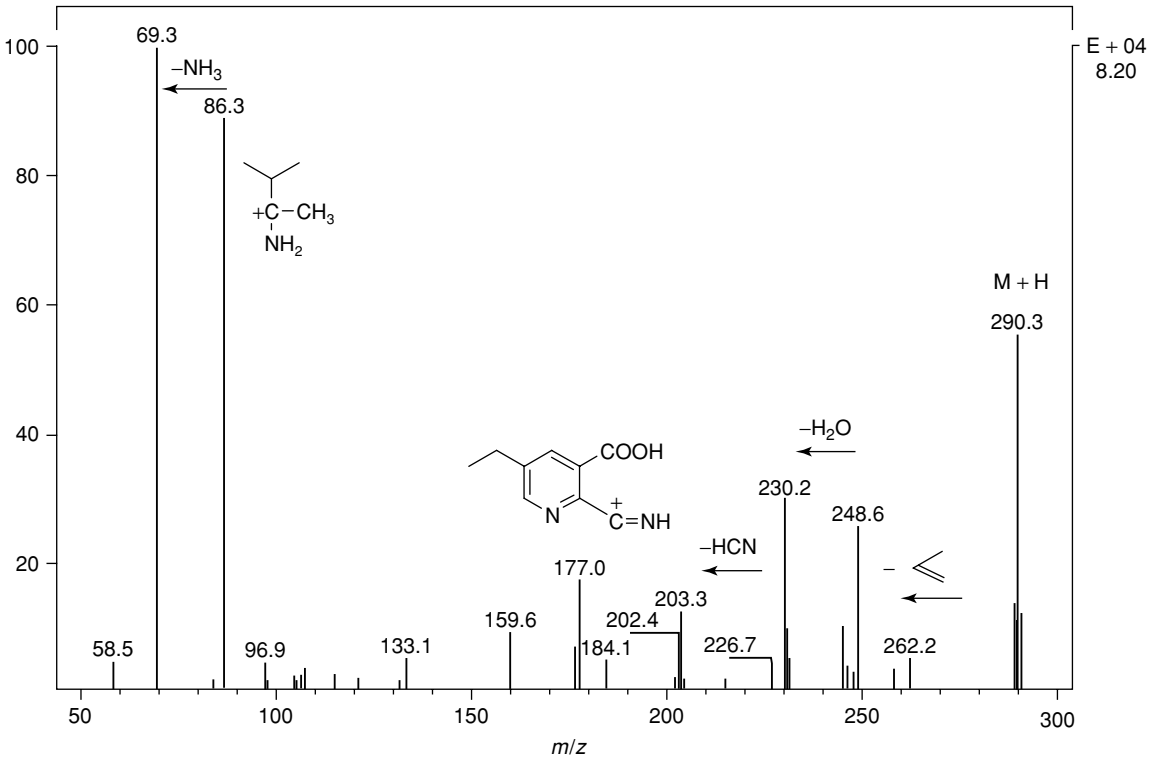


Figure 4 Product ion spectrum from CID of $[M + H]^+$ ion of imazethapyr

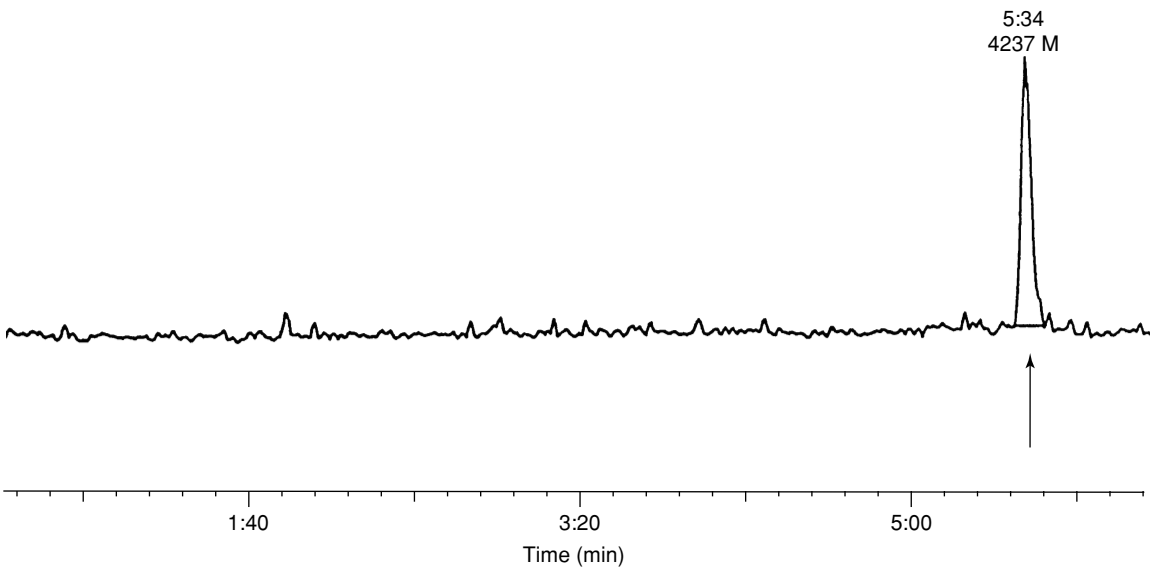


Figure 5 Determination of 1 ng g^{-1} of imazethapyr in soil: HPLC/ESI-MS/MS with SIM of the ion transition m/z 290 → 86

Table 7 List of ions monitored for acidic herbicides (HPLC/ESI-MS)

Analyte	Ions: $[M - H]^-$, and fragment ions, m/z
8-Hydroxybentazone	255, 192
6-Hydroxybentazone	255
Benazolin	198, 170
Bentazone	239
2,4-D	219, 161
MCPA	199, 141
MCPP	213, 141
MCPB	227, 141

involved filtering the water followed by the direct injection of the water sample into the HPLC/MS system (without sample preconcentration). The mobile phase was acetonitrile–0.15% acetic acid in water (1 : 1, v/v). The HPLC column was a Zorbax RX-C8, 2.1 × 150 mm, 5-μm particle size, with a flow rate of 0.2 mL min^{-1} and a 150-μL injection. The limit of quantitation (LOQ) for 2,4-D was 1.0 ng mL^{-1}, which was adequate for the application, and hence post-column addition of 0.1 mL min^{-1} of tripropylamine was not necessary.

3.2.6 N-Methyl carbamate insecticides

The increased use of *N*-methyl carbamate insecticides in agriculture demands the development of selective and sensitive analytical procedures to determine trace level residues of these compounds in crops and other food products. HPLC is the technique most widely used to circumvent heat sensitivity of these pesticides. However, HPLC with UV detection lacks the selectivity and sensitivity needed for their analysis. In the late 1970s and early 1980s, HPLC using post-column hydrolysis and derivatization was developed[38] and refined[39] with fluorescence detection to overcome these problems. The technique relies on the post-column hydrolysis of the carbamate moiety to methylamine with subsequent derivatization to a fluorescent isoindole product. This technique is currently the most widely used HPLC method for the determination of carbamates in water[40] and in fruits and vegetables.[41,42]

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In addition to HPLC/fluorescence, there are references to the use of both APCI and/or ESI with HPLC/MS for the determination of *N*-methyl carbamate insecticides in a variety of matrices.[43–45] Ongoing studies at the US EPA for the determination of *N*-methyl carbamate insecticides in nine fruits and vegetables at the 1.0 ng g^{-1} level are described below. The fruits and vegetables investigated were cranberries, peaches, blueberries, kiwi, carrots, tomatoes, potatoes, lettuce, and grapefruit juice. The purpose of including an account of this work is to illustrate why HPLC/MS/MS is the method of choice for residue work at the 1.0 ng g^{-1} level, especially for difficult matrices.

In this study, HPLC/fluorescence was compared with HPLC/ESI-MS and HPLC/ESI-MS/MS. A summary of the procedure is described as follows. The sample was prepared using the method of Luke *et al.*,[46] and the sample was then cleaned up using a 1-g aminopropyl SPE cartridge. The sample was loaded on to the

Table 8 Ion transition data for 12 *N*-methyl carbamate insecticides (HPLC/ESI-MS/MS)

N-Methyl carbamate	Primary ion transition, *m/z*	Secondary ion transition, *m/z*
Aldicarb sulfoxide	207 → 132	207 → 89
Aldicarb sulfone	223 → 148	223 → 166
Oxamyl[a]	237 → 72	237 → 90
Methomyl	163 → 88	163 → 106
3-Hydroxycarbofuran[a]	238 → 163	238 → 181
Aldicarb[a]	208 → 116	208 → 89
Propoxur	210 → 168	210 → 153
Carbofuran	222 → 165	222 → 123
Carbaryl	202 → 145	202 → 117
Thiodicarb	355 → 88	355 → 163
Isoprocarb	194 → 95	194 → 137
Methiocarb	226 → 121	226 → 169

[a]In the case of aldicarb, oxamyl, and 3-hydroxycarbofuran, the ion transitions go from $[M + NH_4]^+ \rightarrow$ product ions.

cartridge in a 5% dichloromethane–hexane. The SPE cartridge was washed with 5 mL of 5% dichloromethane–hexane, and the analytes were eluted with 10 mL of 1% methanol–dichloromethane. The sample was evaporated to dryness, and the residue was dissolved in and diluted to the appropriate volume with 1 : 1 methanol–pH 3 buffer. Samples were analyzed by HPLC/fluorescence (post-column derivatization) and by HPLC/ESI-MS or HPLC/ESI-MS/MS.

For ESI, the HPLC gradient started with acetonitrile–10 mM ammonium acetate (3 : 17) for the first 3 min and was changed to 9 : 1 acetonitrile–10 mM ammonium acetate in 31 min (held for 4 min). The HPLC column was a Zorbax RX-C8, 2.1-mm i.d. × 150 mm, 5-μm particle size, with a flow rate of 0.15 mL min^{-1} and a 20-μL injection.

For APCI (if matrix effects become a problem in ESI), the mobile phase consisted of (A) 9 : 1 methanol–water containing 50 mM ammonium acetate and (B) water containing 50 mM ammonium acetate–methanol (9 : 1). The gradient was held at 50% A–50% B for 10 min and was then changed to 90% A–10% B in 22 min (held for 3 min). The HPLC column was a Zorbax RX-C8, 4.6-mm i.d. × 250 mm, 5 μm particle size, with a flow rate of 1.0 mL min^{-1} and a 50-μL injection. Table 8 shows the ion transitions (parent to product ions) that were monitored for HPLC/ESI-MS/MS. For single-stage HPLC/ESI-MS, Table 9 shows the ions that were monitored.

In this study, the preliminary findings showed that the HPLC/fluorescence data were in agreement for all 12 carbamates with HPLC/ESI-MS/MS for most of the nine fruits and vegetables at the 1.0 ng g^{-1} fortification level. The recoveries were generally within 70–120%; however, at the 1.0 ng g^{-1} level in each commodity, HPLC/ESI-MS (single-stage MS) had difficulty with interferences for three out of the 12 carbamate pesticides (aldicarb sulfoxide, aldicarb sulfone, and 3-hydroxycarbofuran), which made quantification impossible for these three compounds.

There were also problems with interferences using HPLC/fluorescence with carrots and grapefruit juice for most of the carbamates at the 1 ng g^{-1} level. For example,

Table 9 Parent ions, $[M + H]^+$, monitored for 12 *N*-methyl carbamate insecticides (HPLC/ESI-MS)

N-Methyl carbamate	Parent ion, *m/z*
Aldicarb sulfoxide	207
Aldicarb sulfone	223
Oxamyl[a]	237
Methomyl	163
3-Hydroxycarbofuran[a]	238
Aldicarb[b]	116
Propoxur	210
Carbofuran	222
Carbaryl	202
Thiodicarb	355
Isoprocarb	194
Methiocarb	226

[a]In the case of oxamyl and 3-hydroxycarbofuran,the ion monitored is $[M + NH_4]^+$.
[b] In the case of aldicarb, the ion monitored is $[MH - 75]^+$.

Figure 6 shows the results for a control grapefruit juice sample that gave suspected levels of 3-hydroxycarbofuran using HPLC/fluorescence (a false positive). Other false positives were reported for several of the carbamate insecticides in the control grapefruit juice using HPLC/fluorescence. Figure 7 shows the results for a control grapefruit juice sample (bottom) and a 1.0 ng g^{-1} fortification of 3-hydroxycarbofuran in grapefruit juice (top), using HPLC/ESI-MS. The control in HPLC/ESI-MS also contained reportable levels of 3-hydroxycarbofuran. Figure 8 is similar to Figure 7, except that HPLC/ESI-MS/MS was used. In this case, the potential false positive

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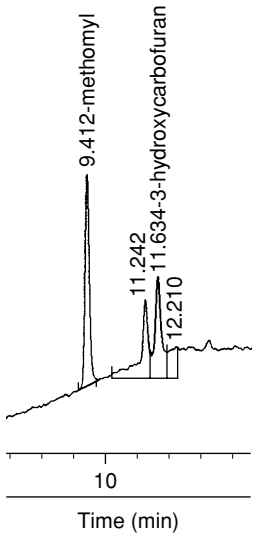


Figure 6 HPLC/fluorescence of grapefruit juice control

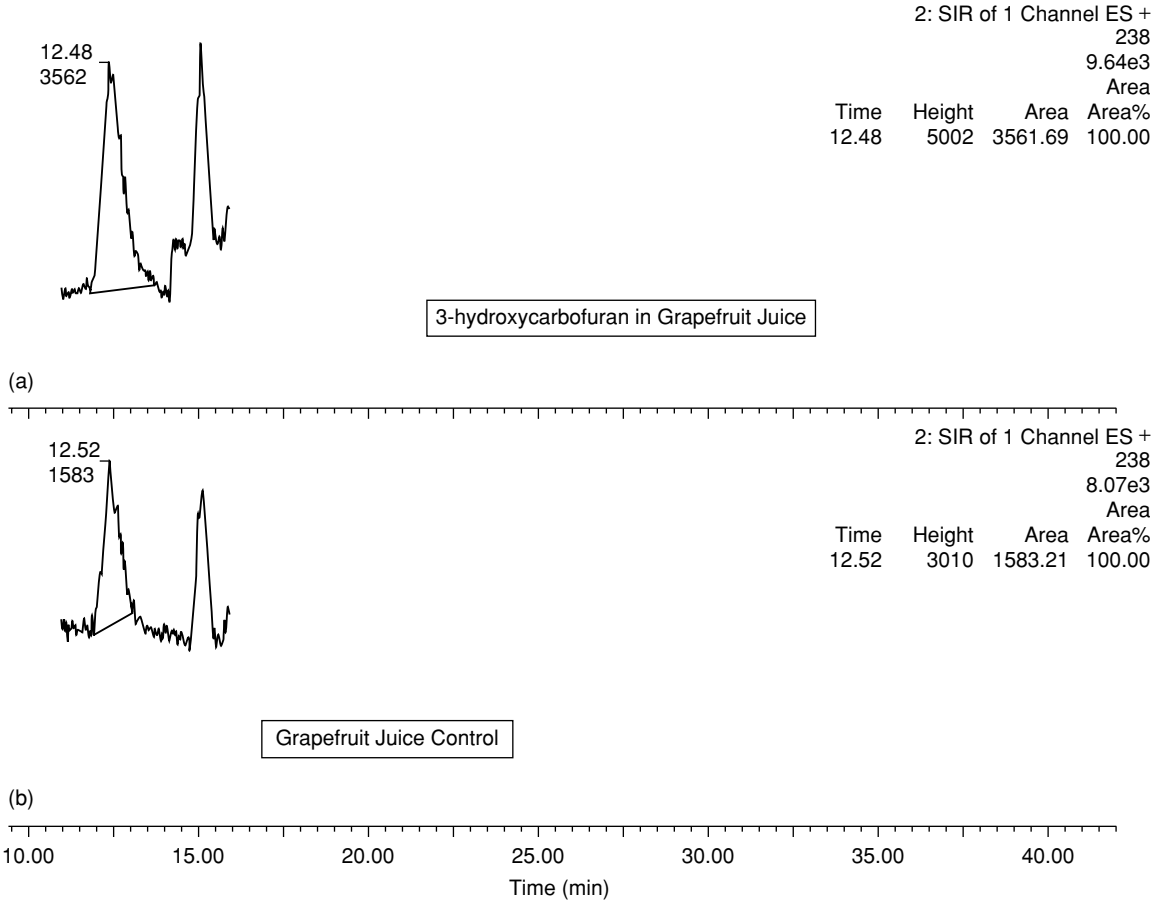
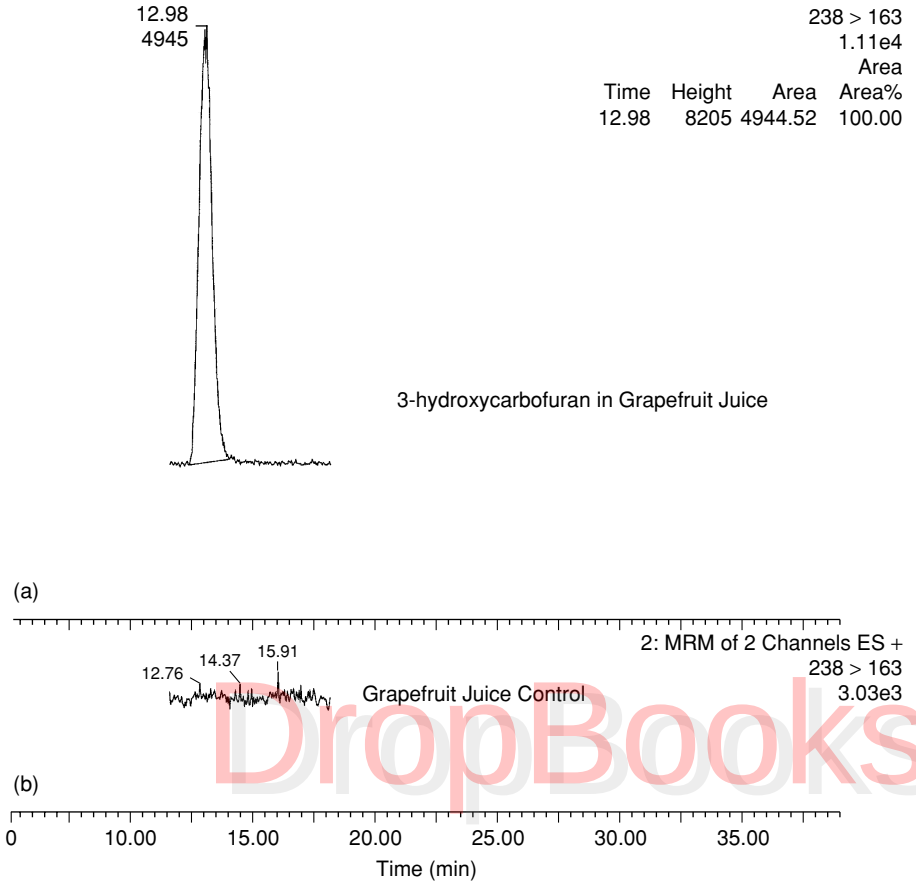


Figure 7 HPLC/ESI-MS of grapefruit juice fortified with 1.0 ng mL^{-1} of 3-hydroxycarbofuran (top) and grapefruit juice control (bottom). Ion monitored: $[M + NH_4]^+$ ion at m/z 238

was avoided. From this example, HPLC/ESI-MS/MS is obviously the preferred technique for analyzing residues at the 1.0 ng g^{-1} level and is, therefore, the primary tool for quantitating the carbamate residues in difficult matrices (e.g., carrots and grapefruit juice).

In the case of carbamate insecticides, both ESI and APCI can be used. However, in this study, the sensitivity of APCI was 3–5-fold less than that of ESI. In this case, the Z-spray configuration was used with APCI, which gives a lower efficiency of ions reaching the mass analyzer than is achieved with other instrumental configurations.

APCI can help to reduce matrix effects when analyzing for carbamate insecticides. For example, when analyzing for methiocarb in citrus products, the apparent recoveries were in the region of 50% with ESI. However, on changing to APCI, the apparent recoveries were increased to 110%. This is an example where APCI can be an alternative API method if matrix effects are a problem with ESI. It is important to note that the analyte must show sufficient sensitivity to both API techniques.



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Figure 8 HPLC/ESI-MS/MS of grapefruit juice fortified with 1.0 ng mL^{-1} of 3-hydroxycarbofuran (top) and grapefruit juice control (bottom). SIM of the ion transition m/z 238 → 163

3.2.7 Matrix effects with HPLC/ESI-MS

Despite the success of electrospray for quantitative analysis, the technique does have certain limitations. One such fundamental problem is a limited dynamic range.[47] Kerbarle and co-workers[17,48] reported a linear response from 10^{-8} to 10^{-5} M for various organic bases. At about 10^{-5} M, the response no longer increases with increase in concentration but levels off and eventually begins to decrease. The cause of the nonlinear response is under investigation by various research groups. Experiments by Bruins[49] indicated that the limited dynamic range is caused by an inability of droplet charge to be converted to gas-phase ions that can be mass analyzed.

As already mentioned, a challenge in the application of ESI, at least for quantification, is ion suppression. Another example of matrix effects is observed in the

Table 10 Summary of literature references for HPLC/MS applications of various classes of pesticides

Compound class	API mode	Reference
Sulfonylurea herbicides	ESI	54–61
Imidazolinone herbicides	ESI	4, 29, 32, 34, 35, 62
N-Methyl carbamate insecticides	APCI and ESI	43–45
Triazine herbicides	APCI and ESI	27, 28, 63–66

determination of pyrithiobac sodium [sodium 2-chloro-6-(4,6-dimethoxypyrimidin-2-yl)benzoate] in cotton gin trash by HPLC/ESI-MS/MS. When using external standards, the recoveries ranged from 50 to 55%, and recoveries for control extracts fortified just prior to injection ranged from 60 to 70%. Low recoveries for extracts fortified just prior to analysis indicate that matrix suppression occurred in the electrospray source.[50,51] Although MS/MS removed co-eluting compounds from the baseline, matrix effects were not reduced. Matrix effects result from changes in ionization efficiency due to competition for charge at the droplet level.[52]

In an attempt to minimize matrix effects, the APCI interface was used to analyze samples using the same purification procedure (PLE followed by liquid–liquid partitioning). In APCI, the method generated acceptable recoveries (70–120%) at the 20 and 40 ng g^{-1} levels.

The APCI interface was less susceptible to matrix suppression than was the electrospray interface for pyrithiobac sodium in cotton gin trash.[53] The corona discharge in the APCI source appeared to produce enough charge to ionize all compounds present at any given time regardless of the presence of co-eluting compounds. The thermal stability of pyrithiobac sodium enabled it to withstand the elevated temperature of the APCI interface without significant thermal degradation. However, thermally labile compounds often do not give sufficient response for low-level quantification by APCI. Also, in contrast to the signal suppression observed in ESI, signal enhancement is occasionally observed in APCI.

Other ways to minimize matrix effects include improving the sample cleanup, diluting the sample, using labeled internal standards, using standard addition, or using matrix-matched standards. The last approach, however, is not permitted for enforcement methods at present by the US EPA or the US Food and Drug Administration (FDA).

Table 10 gives a summary of references along with the ionization modes used for the various classes of pesticides. These classes include those discussed earlier, together with other classes not discussed. We hope that this information will provide guidance for applying HPLC and API-MS successfully to the compounds of interest. Instrumentation for HPLC/MS and HPLC/MS/MS has become more commercially available, and its cost is more affordable for enforcement laboratories than it was several years ago. Although an MS instrument is still initially a more expensive and complex device than most other LC detectors, once the instrument is up and running, MS can be very dependable and reliable. MS can then eliminate much time-consuming effort in sample analysis and method development. For residue work at or below the 10 ng mL^{-1} level, it is strongly recommended that LC/MS/MS be used.

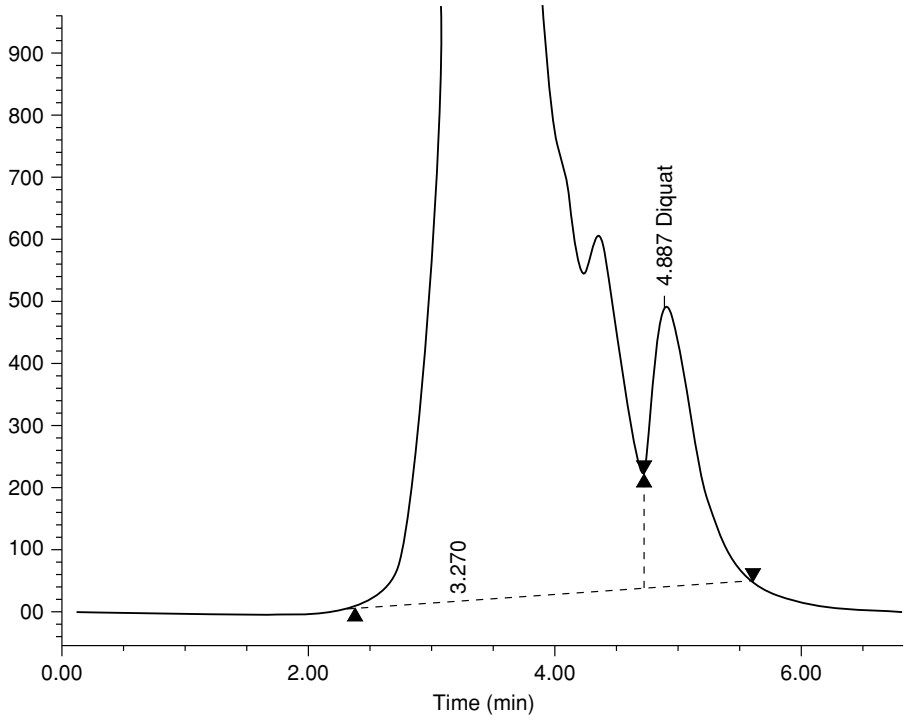


Figure 9 HPLC chromatogram of a diquat standard with UV detection

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3.3 Capillary electrophoresis (CE)

A variety of formats and options for different types of applications are possible in CE, such as micellar electrokinetic chromatography (MEKC), isotachophoresis (ITP), and capillary gel electrophoresis (CGE). The main applications for CE concern biochemical applications, but CE can also be useful in pesticide methods.[57,62,67–72] The main problem with CE for residue analysis of small molecules has been the low sensitivity of detection in the narrow capillary used in the separation. With the development of extended detection pathlengths and special optics, absorbance detection can give reasonably low detection limits in clean samples. However, complex samples can be very difficult to analyze using capillary electrophoresis/ultraviolet detection (CE/UV). CE with laser-induced fluorescence detection can provide an extraordinarily low LOQ, but the analytes must be fluorescent with excitation peaks at common laser wavelengths for this approach to work. Derivatization of the analytes with appropriate fluorescent labels may be possible, as is done in biochemical applications, but pesticide analysis has not been such an important application to utilize such an approach.

As in HPLC, the coupling of MS detection with CE has provided an excellent opportunity for more selective analysis, but the much reduced flow rates, small injection volumes, limitations in the types of buffers used [since electrospray ionization (ESI) is used in capillary electrophoresis/mass spectrometry (CE/MS)], and need to

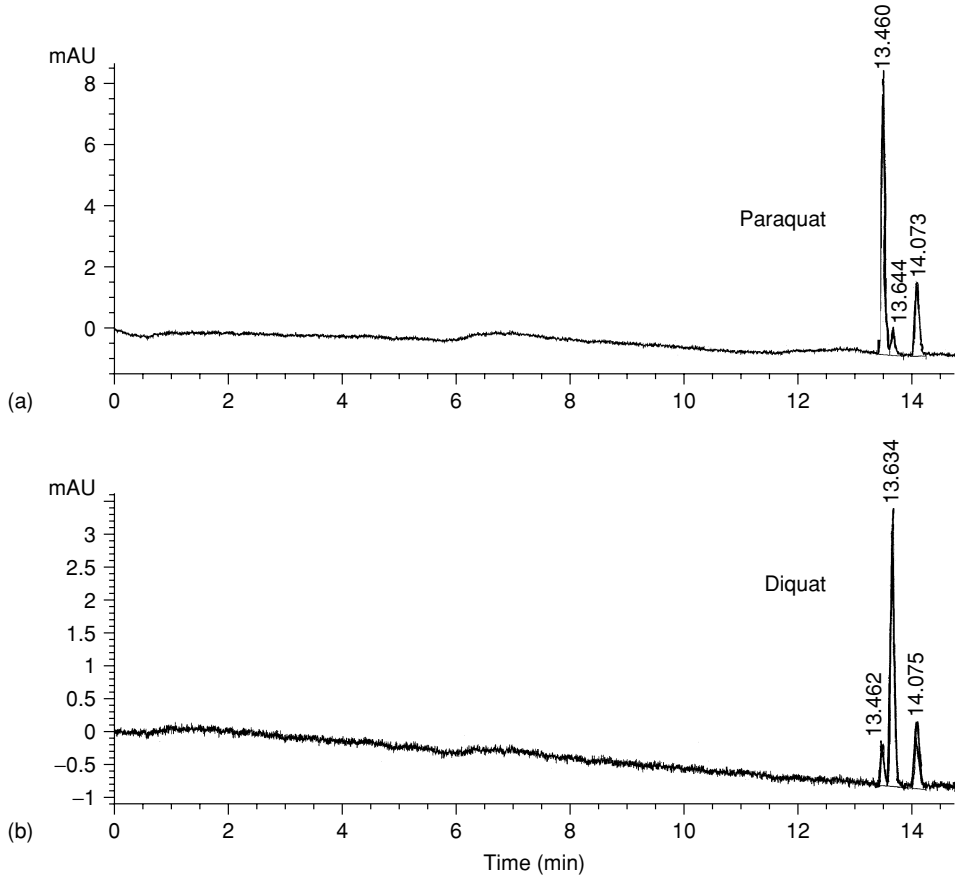
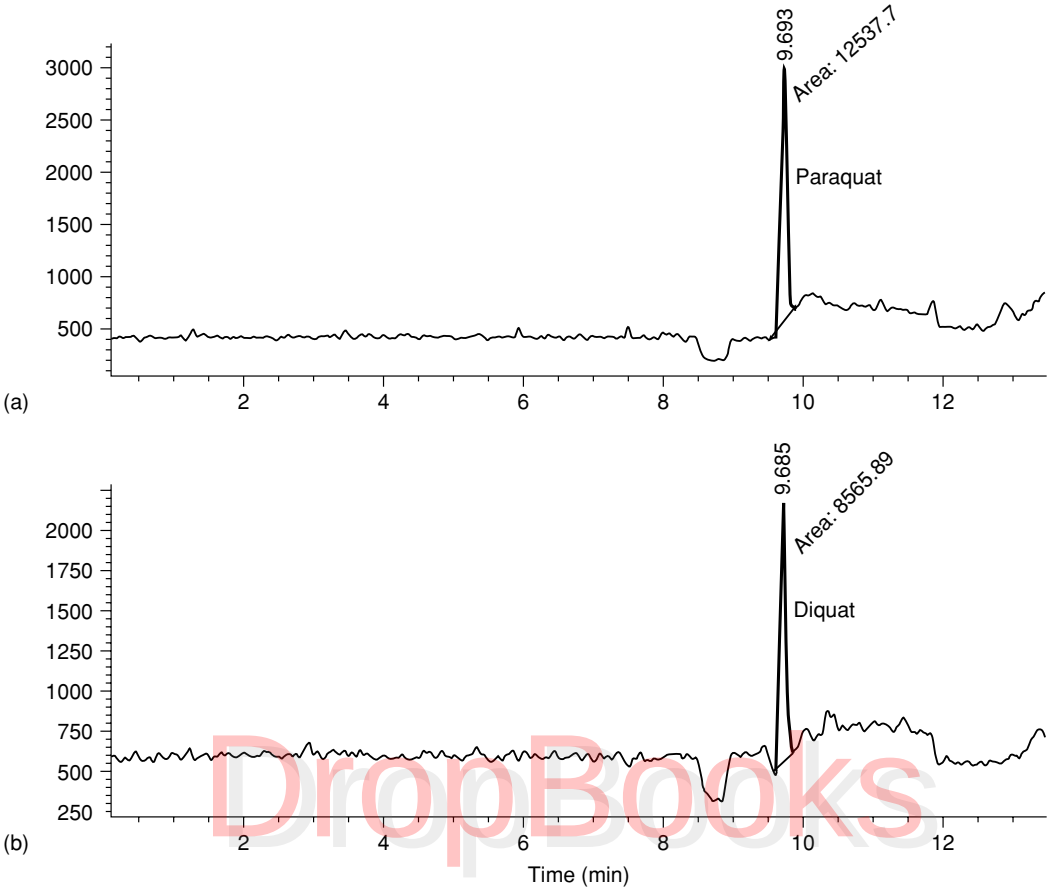


Figure 10 CE/UV of alfalfa hay fortified with 0.25 mg kg^{-1} of paraquat (upper half) and 0.28 mg kg^{-1} of diquat (lower half). Buffer: 50 mM phosphate at pH 2.5. The wavelength monitored was 258 nm for paraquat and 310 nm for diquat

maintain a large voltage potential along the capillary make CE/MS more complicated than HPLC/MS. Furthermore, 1000-fold lower injection volumes in CE lead to higher LOQ in CE/MS than in HPLC/MS. Techniques such as stacking multiple CE injections can increase injection volumes, possibly to overcome the sample injection volume limitation in CE, but HPLC/MS can often achieve the same results with less trouble than CE/MS.

A limited number of 'in-house' experiments at the EPA (unpublished work) were performed in the comparison of high-performance liquid chromatography/ultraviolet detection (HPLC/UV), CE/UV, and CE/MS on the feasibility of analyzing alfalfa hay extracts for paraquat and diquat. Figure 9 shows an HPLC/UV chromatogram of a diquat standard, and Figure 10 shows a CE/UV electropherogram of an alfalfa hay sample fortified with 0.25 mg kg^{-1} of paraquat and 0.28 mg kg^{-1} of diquat. Figure 11 shows a CE/MS electropherogram of the same alfalfa hay extract fortified at the same concentrations. CE/UV gives the best performance of the three methods. Although the two compounds were separated by mass, the CE/MS electropherogram using acetate buffer gave a worse separation than the CE/UV



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Figure 11 CE/MS of alfalfa hay fortified with 0.25 mg kg^{-1} of paraquat (upper half) and 0.28 mg kg^{-1} of diquat (lower half). Buffer: 50 mM ammonium acetate at pH 4.5. Sheath liquid: methanol–water–50 mM ammonium acetate (5 : 4 : 1). Positive ESI

electropherogram. Unfortunately, the phosphate buffer that gave the better separation with CE/UV cannot be used in CE/MS. Also, the S/N ratio with CE/MS was poor owing to the small injection volume of only 70 nL. Other similar work on the determination of paraquat and diquat by CE/MS has been published elsewhere.[72]

The main advantages of CE over HPLC relate to the greater number of theoretical plates, reduction in the use of hazardous solvents, lack of waste, utility in sample limited applications, and general capability for lower LOQ with UV and fluorescence detectors (owing to increased resolution in separations and higher S/N ratio despite the smaller injection volume). Unlike the laminar flow profile of chromatographic techniques, plug flow occurs in CE because flow originates along the capillary walls. This generally translates into sharper peaks in CE and better separations.

A common criticism of CE is the poor consistency of migration times for peaks compared with HPLC or GC. The effects of matrix components and small differences in ionic strength and pH can have significant effects in the separation. The use of a

migration time marker provides a consistent relative migration time that may be used to identify peaks of interest, especially in complex electropherograms. As in HPLC, separation of a diverse range of pesticides is often difficult in CE with the same set of conditions. Thus, CE applications are most likely to be limited to single-class multiresidue methods. CE is most useful for ionic pesticides, but neutral analytes are also possible using CEC and MEKC. In recent years, certain agrochemical companies have been using CE/UV as an interim method until HPLC/MS was considered accepted methodology in enforcement laboratories.[34,62] The CE methods that were submitted to the EPA OPP were methods primarily for the imidazolinone class of herbicides in soils (LOQ 3 ng g^{-1}) and food commodities (LOQ 600 ng g^{-1}).

4 Conclusions

Owing to the introduction of a number of sophisticated technologies and instruments, tremendous improvements in the ability to analyze multiple pesticides for multiple classes in a variety of sample matrices have occurred in recent years. A growing number of techniques are available to the analytical chemist, and many strategies are possible to meet the purpose of analyses. In general, the use of the fewest analytical steps that provide reliable results in a rugged approach serves as the best overall approach to determining pesticide residues in food, environmental, and other types of samples. An inherent difficulty in multiclass, multiresidue analysis is that as the range of analytes increases, the overall selectivity of the sample preparation decreases. Therefore, trade-offs and compromises must often be made in obtaining high recoveries of a wide range of analytes while minimizing time, effort, and cost of the procedure(s).

Ultimately, these fundamental and practical aspects may limit the ability of the strategy to meet the needs of the analysis. A host of strategies is available to the chemist, but practical concerns in the laboratory, such as time, budgets, available instruments, and personnel, limit the amount of effort and resources that can be devoted to the analysis. This article emphasized the most rugged and useful methods for the greatest number of pesticide applications. This entailed liquid-based extraction, liquid–liquid partitioning and/or SPE cleanup (GPC for fatty samples) and either GC or HPLC as the analytical separation with MS (or MS/MS) used for the simultaneous quantitation and confirmation of analytes. We feel that these time-tested and reliable approaches give the greatest chance of success in developing a new, rugged analytical method. Alternative approaches have become available to the analyst, but these methods have a difficult challenge to achieve better results and greater efficiency than the widely applicable and rugged approaches currently used in pesticide residue analysis.

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References

1. S.J. Lehotay, 'Multi-class, multi-residue analysis of pesticides, strategies for', in "Encyclopedia of Analytical Chemistry: Instrumentation and Applications", ed. R. A. Meyers, John Wiley & Sons Ltd, Chichester, pp. 6344–6384 (2000).
2. S.J. Lehotay and F.J. Schenck, 'Multiresidue Methods: Extraction', in "Encyclopedia of Separation Science", ed. I. D. Wilson, T. R. Adlard, C. F. Poole, and M. Cooks, Academic Press, London, pp. 3409–3415 (2000).
3. A. Fajgelj and A. Ambrus (eds), 'Principles and Practices of Method Validation', Royal Society of Chemistry, Cambridge, 2000.
4. S.J. Stout, *et al. J. Agric. Food Chem.,* **44**, 2182 (1996).
5. F.Q. Bramble, S. Frizzell, S.W. George, B.A. Peterson, D.D. Ranken, J.J. Stry, and A.R. Sumpter, DuPont Project Identification AMR 3954-96: Proposed Analytical Enforcement Method For the Determination of Pyrithiobac Sodium in Cotton Gin Trash Using ASE Extraction and LC/MS/MS Analysis, Dupont, Wilmington, DE (1997).
6. S.J. Lehotay, *J. AOAC Int.,* **85**, 1148 (2002).
7. M. Anastassiades, S.J. Lehotay, D. Stajnbaher, and F.J. Schenck, *J. AOAC Int.*, in press.
8. P.L. Alferness and L.A. Wiebe, *J. AOAC Int.,* **84**, 823 (2001).
9. S. Dagan, *J. Chromatogr. A,* **868**, 229 (2000).
10. M.M. van Deursen, J. Beens, H.-G. Janssen, P.A. Leclercq, and C.A. Cramers, *J. Chromatogr. A,* **878**, 205 (2000).
11. K. Maštovská, S.J. Lehotay, and J. Hajšlová, *J. Chromatogr. A,* **926**, 291 (2001).
12. M. Kochman, A. Gordin, P. Goldshlag, S.J. Lehotay, and A. Amirav, *J. Chromatogr. A,* in press.
13. F. Andre, K.K.G. De Wasch, H.F. De Brabander, S.R. Impens, L.A.M. Stolker, L. van Ginkel, R.W. Stephany, R. Schilt, D. Courtheyn, Y. Bonnaire, P. Furst, P. Gowik, G. Kennedy, T. Kuhn, J.-P. Moretain, and M. Sauer, *Trends Anal. Chem.,* **20**, 435 (2001).
14. C.M. Whitehouse, R.N. Dreyer, Y. Yamashita, and J.B. Fenn, *Anal. Chem.,* **57**, 675 (1985).
15. R.D. Smith, J.A. Loo, C.G. Edmonds, C.J. Barringa, and H.R. Udseth, *Anal. Chem.,* **62**, 882 (1990).
16. J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, and C.M. Whitehouse, *Mass Spectrom. Rev.,* **9**, 37 (1990).
17. P. Kebarle and L. Tang, *Anal. Chem.,* **65**, 972A (1993).
18. J.B. Fenn, J. Rosell, and C.K. Meng, *J. Am. Soc. Mass Spectrom.,* **8**, 1147 (1997).
19. S.J. Gaskell, *J. Mass Spectrom.,* **32**, 677 (1997).
20. C. Enke, *Anal. Chem.,* **69**, 4885 (1997).
21. A.P. Bruins, *J. Chromatogr. A,* **794**, 345 (1998).
22. D. Barcelo (ed.), '*Journal of Chromatography Library*', Elsevier, Amsterdam, Vol. 59 (1996).
23. R.B. Cole (ed.), 'Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation, and Applications', John Wiley & Sons Inc., New York, pp. 3–65 (1997).
24. D.A. Volmer and D.L. Volmer, *LC-GC,* **14**, 236 (1996).
25. P.J. Arpino, *Mass Spectrom. Rev.,* **8**, 35 (1989).
26. R.C. Willoughby and R.F. Browner, *Anal. Chem.,* **56**, 2626 (1984).
27. E.M. Thurman, I. Ferrer, and D. Barcelo, *Anal. Chem.,* **73**, 5441 (2001).
28. E.M. Thurman, I. Ferrer, and D. Barcelo, 'The ionization-continuum diagram: a concept for selection of APCI and ESI conditions for HPLC/MS of pesticides', in "17th Montreux Symposium on Liquid Chromatography/Mass Spectrometry, Montreux, Switzerland, November 8–10, 2000", p. 31 (2000).
29. S.J. Stout, A.R. da Cunha, G.L. Picard, and M.M. Safarpour, *J. AOAC Int.,* **81**, 685 (1998).

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30. J.M. Devine, in 'The Imidazolinone Herbicides', D. L. Shaner and S. L. O'Connor (eds), CRC Press, Boca Raton, FL, Chapter 13 (1991).
31. M.J.M. Wellos and J.L. Michael, *J. Chromatogr. Sci.,* **25**, 345 (1987).
32. S.J. Stout, A.R. da Cunha, G.L. Picard, and M.M. Safarpour, *J. Agric. Food Chem.,* **44**, 3548 (1996).
33. R.D. Mortimer and D.F. Weber, *J. AOAC Int.,* **80**, 426 (1993).
34. A.J. Krynitsky, S.J. Stout, H. Nejad, and T.C. Cavalier, *J. AOAC Int.,* **82**, 956 (1999).
35. S.J. Stout, A.R. da Cunha, and M.M. Safarpour, *J. AOAC Int.,* **80**, 426 (1997).
36. K.N. Reddy and M.A. Locke, *Weed Sci.,* **42**, 249 (1994).
37. S. Chiron, S. Pipilloud, W. Haerdi, and D. Barcelo, *Anal. Chem.,* **67**, 1637 (1995).
38. H.A. Moye, S. Scherer, and P.A. St. John, *Anal. Lett.,* **10**, 1049 (1997).
39. R.T. Krause, *J. Chromatogr. Sci.,* **16**, 281 (1978).
40. K.M. Hill, R.H. Hollowell, and L.A. Dal Cortivo, *Anal. Chem.,* **56**, 2465 (1984).
41. R.T. Krause and E.M. August, *J. AOAC Int.,* **66**, 234 (1983).
42. R.T. Krause, *J. AOAC Int.,* **63**, 1114 (1980).
43. S. Pleasance, J.F. Anacleta, M.R. Baily, and D.H. North, *J. Am. Soc. Mass Spectrom.,* **3**, 378 (1992).
44. A. Di Corcia, C. Crescenzi, A. Lagana, and E. Sebastini, *J. Agric. Food Chem.,* **44**, 1930 (1996).
45. D.A. Volmer, D.L. Volmer, and J.G. Wilkes, *LC-GC,* **14**, 216 (1996).
46. M.L. Luke, J.E. Froberg, and H.T. Masumoto, *J. AOAC Int.,* **58**, 1020 (1975).
47. D.L. Buhrman, P.I. Price, and P.J. Rudewicz, *J. Am. Soc. Mass Spectrom.,* **7**, 1099 (1996).
48. M.G. Ikonomou, A.T. Blades, and A.T. Kerbarle, *Anal. Chem.,* **62**, 957 (1990).
49. A.P. Bruins, Presented at the 7th Sanibel Conference on Mass Spectrometry, Sanibel Island, FL, January 21–24, 1995.
50. C.G. Enke, *Anal. Chem.,* **69**, 4885 (1997).
51. B.K. Matuszewski, M.L. Constanzer, and C.M. Chavez-Eng, *Anal. Chem.,* **70**, 882 (1998).
52. A.T. Blades, M.G. Ikonomou, and P. Kerbarle, *Anal. Chem.,* **63**, 2109 (1991).
53. W.J. Blanchflower, P.J. Hughes, and D.G. Kennedy, *J. AOAC Int.,* **80**, 1177 (1997).
54. M. Rodriguez and D.B. Orescan, *Anal. Chem.,* **70**, 2710 (1998).
55. R. Bossi, B. Koppen, N.H. Splüd, and J.C. Streibeg, *J. AOAC Int.,* **81**, 775 (1998).
56. A. Di Corcia, C. Crescenzi, R. Sampari, and L. Scappaticcio, *Anal. Chem.,* **69**, 2819 (1997).
57. J.J. Stry, J.S. Amoo, S.W. George, T. Hamilton-Johnson, and E. Stetser, *J. AOAC Int.,* **83**, 651 (2000).
58. A.J. Krynitsky, *J. AOAC Int.,* **80**, 392 (1997).
59. L.J. Marek and W.C. Koskinen, *J. Agric. Food Chem.,* **44**, 3978 (1996).
60. D. Volmer, J.G. Wlikes, and K. Levsen, *Rapid Commun. Mass Spectrom.,* **9**, 767 (1995).
61. C.R. Powley, 'Sulfonylurea herbicides', in "Handbook of Residue Analytical Methods for Agrochemicals", ed. P. W. Lee, John Wiley & Sons Ltd, Chichester, Vol.1, (2003).
62. H. Nejad, M.M. Safarpour, T. Cavalier, G. Picard, M. Souza, A.J. Krynitsky, S. Chiv, P. Miller, and S. Stout, *J. Capillary Electrophoresis,* **5**, 81 (1998).
63. C. Crescenzi, G. D'Ascenzo, A. Di Corcia, M. Nazzari, S. Marchese, and R. Samperi, *Anal. Chem.,* **71**, 2157 (1999).
64. A. Di Corcia, C. Crescenzi, E. Guerriero, and P. Samperi, *Environ. Sci. Technol.,* **31**, 1658 (1997).
65. E.M. Thurman and C. Batian, 'Determination of atrazine and atrazine mercapture in drinking water samples and in urine using immunoaffinity SPE with positive ion spray HPLC/MS', Presented at the 15th Symposium on Liquid Chromatography/Mass Spectrometry, Montreux, Switzerland, November 9–10, 1998.
66. I. Ferrer, E.M. Thurman, and D. Barcelo, *Environ. Sci. Technol.,* **34**, 714 (2000).
67. Z. El Rassi, *Electrophoresis,* **18**, 2465 (1997).
68. A.J. Krynitsky and D.M. Swineford, *J. AOAC Int.,* **78**, 1091 (1995).
69. F. Garcia and J. Henion, *J. Chromatogr. A,* **606**, 237 (1992).
70. G.M. McGaughlin, A. Weston, and K.D. Hauffe, *J. Chromatogr. A,* **744**, 123 (1996).
71. S.C. Beale, *Anal. Chem.,* **70**, 279R (1998).
72. X. Song and W.L. Budde, *J. Am. Soc. Mass Spectrom.,* **7**, 981 (1996).

Best practices in the generation and analyses of residues in environmental samples

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Best practices in the analysis of residues in environmental samples: groundwater and soil-water monitoring procedures

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1 Introduction

This article provides an overview of methodology and materials for developing groundwater sampling procedures to investigate the presence of pesticides in groundwater. 'Pesticide' is used as a general term to represent chemicals such as herbicides, insecticides, fungicides, and related transformation products. The presence of pesticides in groundwater may be the result of point source releases to the environment and/or nonpoint source use for the production of agricultural commodities, for residential pest and weed control, or for pest eradication programs.

The goal of groundwater sampling is to obtain samples that are representative of the aquifer being investigated and to minimize the bias introduced by the methods employed to collect those samples. The purpose of the collection of representative samples is to investigate whether pesticide products in groundwater are present and, if present, at what concentration.

The design of groundwater monitoring procedures is based on the objectives of the water-quality investigation. The selected procedures comprise a monitoring plan and commonly include: the selection of sampling points (either national, regional, or field scale); the determination of appropriate sources for sampling (monitoring well or water supply well); the selection of field equipment and supplies compatible with the compound(s) of interest and the objectives of the study; and the establishment of field procedures and quality control (QC) procedures for sample collection and handling. The implementation of a groundwater monitoring plan is discussed.

In addition to groundwater sampling, water-quality investigations often monitor the potential migration of pesticides through the unsaturated zone. Suction lysimeters are instruments suitable for monitoring pesticide transport through the unsaturated zone and are an important component of some field-scale groundwater monitoring studies.[1] Suction lysimeter installation and sampling are described.

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2 Sources for the collection of groundwater samples

A groundwater monitoring plan identifies where groundwater samples will be collected. Both horizontal spacing between sampling points and vertical spacing within the aquifer are specified. For a geographic area under investigation, a number of potential sampling points may already exist. These could include monitoring wells from other investigations or water supply wells for domestic (homeowner), municipal, public, industrial, or irrigation uses. Other potential sampling points may include springs, seeps, and drain tiles.

As part of developing the groundwater monitoring plan, the potential availability and suitability of these sampling points should be considered. Initially, permission to sample the groundwater source must be granted by the owner. In the case of wells, once approval has been granted, well construction, age, use history, integrity, location, and design specifications must be collected, documented, and interpreted to determine the well's suitability given the goals of the monitoring plan. Owners or drillers may be able to provide the required details. Field inspection of the well (by using sounding devices, observation, borehole cameras, and geophysical logging tools) can establish or confirm several important characteristics (e.g., total depth, screen depth ranges, surface completion, security, etc.). Also, many regulatory agencies can provide documentation about wells under their jurisdiction.

The suitability inspection often eliminates a large number of potential sampling points from consideration. Selected wells must be pumping groundwater from the correct water-bearing zone of the aquifer under investigation. Samples will need to be collected as close to the wellhead as possible and before entering a treatment system or delivery system (not always possible without modification). If a sufficient number of existing sampling points are not available to meet the requirements of the groundwater monitoring plan, monitoring wells may need to be installed.

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2.1 Monitoring wells

Groundwater monitoring wells are specifically designed to provide access to groundwater and to permit the investigation of prescribed aspects of the surrounding subsurface environment. Groundwater monitoring wells can be installed for a number of investigative tasks, including:

- the collection of groundwater samples for analysis
- the collection of light (lighter than water), nonaqueous-phase liquids (LNAPL)
- the collection of dense (heavier than water) nonaqueous-phase liquids (DNAPL)
- determining the elevation of the water table
- determining the potentiometric water level within an aquifer
- testing aquifer permeability
- the collection of soil gas
- providing access for geophysical logging tools.

A single monitoring well can be designed to accommodate all of these uses and wells are usually designed to collect only those data required by the groundwater monitoring plan.

Although a number of well types can be used for monitoring groundwater, a monitoring well is designed for collecting specific groundwater data. A conventional monitoring well is comprised of a section of pipe (screen) installed in the ground that has openings (slots) to permit the entry of groundwater, above which is connected a section of solid pipe (casing) which extends to the surface. If the screened interval of the well is to be installed through a competent bedrock formation, use of a screen can be omitted and the resulting well is referred to as an open-hole well.[2] Figure 1 provides a schematic diagram of a conventional screened well and an open-hole bedrock well. A porous inert substance (i.e., silica sand) is installed to surround the screen section and the casing above the screen is sealed in the borehole by grout. The void between the well and the borehole wall is termed the annular space. A locking protective cover is installed at the ground surface to complete the installation.

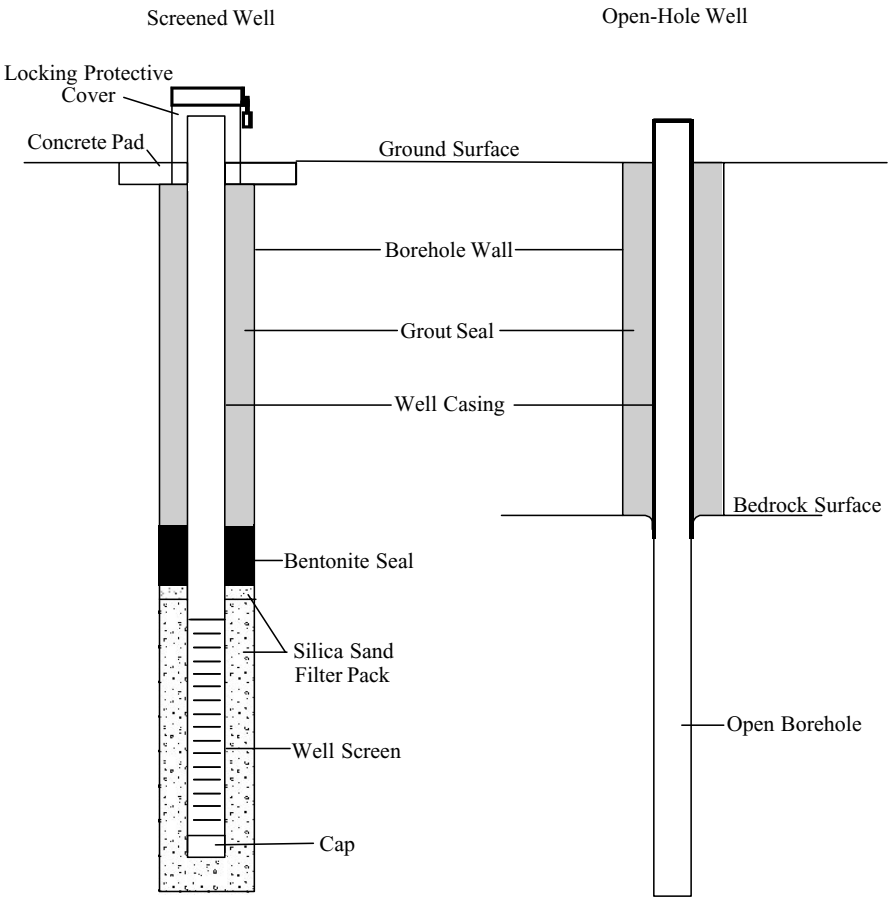


Figure 1 Generalized construction of a screened well and an open-hole well

2.1.1 Monitoring well installation

The installation of a monitoring well or group of monitoring wells entails many of the same activities as a small construction project. Research is conducted to develop an understanding of the subsurface conditions which may be encountered. Preliminary well construction plans are prepared to accommodate the expected subsurface conditions. Regulatory approvals (permits) need to be applied for and received before installation can begin in some jurisdictions. Property owner approval, typically in the form of a lease arrangement, needs to be acquired if the investigator does not own the property. Negotiations should begin well in advance of field activities. Underground utilities must be located before any subsurface work commences. Obstructive overhead power lines, right-of-way restrictions, set-back distances, property line locations, etc., must also be identified and wells situated accordingly. Access to a supply of potable water, collection and disposal of waste materials (especially if they are expected to be contaminated), a safe, secure, clean storage area for well supplies, and other standard construction project preparations need to be completed.

2.1.2 Drilling methods

To install a conventional groundwater monitoring well, a vertical hole is dug to a prescribed depth below the water table. For some shallow wells, the hole can be excavated by hand with a bucket auger. While deepening the borehole, it is important to record a description of the composition and characteristics of the formation materials encountered to provide a record of the subsurface. Samples from the bucket auger can also be packaged according to their discrete depth interval and sent for laboratory analysis. If well depth requirements exceed the limits of the bucket auger or formation collapse prevents completing the hole to the appropriate depth, a number of other drilling methods may be employed.

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Drilling machines provide a number of benefits over using a hand-operated bucket auger. They can be faster than working by hand and can advance a borehole through almost all subsurface environments (including bedrock). It is also possible to combine different drilling methods to work in complex subsurface conditions. Drilling machines can operate a variety of soil and bedrock sampling equipment some of which can collect relatively undisturbed formation samples [e.g., split-spoon (split-barrel) sampler, thin-wall (Shelby) open-tube sampler, core barrel sampler].[3] In addition, drilling machines install a temporary, retractable casing. This temporary casing provides a relatively unobstructed borehole to install the monitoring well materials while preventing or limiting formation collapse.

Common drilling methods include solid-stem augering, hollow-stem augering, and water-, air-, and mud-rotary. These drilling methods can be divided into two categories based on how they remove the materials they drill through (cuttings). Hollow-stem and solid-stem augers utilize spiral flights to lift cuttings to the surface. Other drilling methods introduce a fluid or compressed air to lift the cuttings and remove them from the borehole (e.g., water-, mud-, and air-rotary methods). Both drilling methods may change the natural subsurface environment to some extent, which may in turn affect the collection of representative groundwater samples. Selecting the most appropriate

method depends on the subsurface conditions and the specific needs of the monitoring well to be installed.

Although both categories may alter the subsurface environment, the extent to which they do this differs. Hollow-stem and solid-stem augers may change the formation materials in the immediate vicinity of the well by forcing the cuttings into the borehole wall while lifting them to the surface. This may create a smear zone along the borehole wall because fine-grained particles can be pressed into voids in the formation or the formation materials may become more tightly packed, effectively reducing existing permeability and lowering the flow rate of water to the well. The transfer of the cuttings upward by the auger flights may also move contaminated cuttings from one area of the borehole to another. For the most part, the effects of auger drilling are normally restricted to the immediate vicinity of the borehole wall.

Water-, mud-, and air-rotary drilling methods may force cuttings, drilling fluids or compressed air into the voids in the borehole wall. The result may be similar to that of auger drilling in that the natural permeability of the formation in the vicinity of the borehole wall may be reduced, and contaminated materials may be spread to formerly uncontaminated areas. However, the result may be more extensive than auger drilling because the distance the cuttings, drilling fluids, and compressed air migrate into the formation may be greater. If the natural permeability of the formation is high enough, these materials will be able to migrate beyond the immediate vicinity of the borehole wall. The extent of movement into the surrounding formation is difficult to quantify.

Given these drilling method characteristics, shallow wells installed in unconsolidated or poorly indurated formations are most commonly completed with hollow-stem auger drilling methods. Solid-stem augers can only be used where the formation materials are cohesive enough not to collapse during well installation. Wells that exceed the working depth of auger rigs or wells to be completed in bedrock are installed with water-, mud-, or air-rotary drilling methods. Because these methods introduce foreign materials into the borehole, they are not as desirable as auger drilling methods.

2.1.3 Well types

The two types of groundwater monitoring wells are screened and open hole (Figure 1). Screened wells have a slotted section of pipe below the casing to permit the entry of groundwater. Open-hole wells have no slotted pipe below the casing but rather finish in a section of open borehole. Open-hole well construction may be selected for monitoring groundwater in competent bedrock. However, bedrock formations that are prone to deteriorate over time (e.g., weakly indurated sandstones) or have a fracture pattern that would result in damage to the well would not be candidates for open-hole monitoring wells. If the competency of the bedrock is uncertain, a screen section or a screened well can be constructed in the bedrock.

Groundwater monitoring plans for pesticide investigations commonly require groundwater samples from, at a minimum, the uppermost water-bearing zones of the subsurface. However, for some monitoring plans, groundwater samples are required from more than one depth at each location. Plans may specify multiple wells at each sampling location (so-called well clusters or nested wells) or multiple sampling depths within a single well. Aller *et al.*[4] describe a number of well configuration options to accomplish these requirements. Screened wells are the most appropriate

well type where the uppermost water-bearing zone resides in unconsolidated or poorly consolidated sediments or unstable bedrock formations.

2.1.4 Well construction materials

Materials used to construct monitoring wells are selected while preparing the groundwater monitoring plan. The overriding concern is that the selected materials be as inert as possible in relation to the substances being monitored while being compatible with the expected subsurface conditions. Well materials include the screen and riser pipe, the filter pack, and the grout.

Well construction materials should be delivered to the installation site clean and preferably individually packaged by the manufacturer. The manufacturer should warrant their materials to be clean upon delivery. Damaged or opened packaging should not be accepted for installation. Decontamination or pre-installation cleaning of materials may be necessary. A pressure washer capable of providing heated water or steam is typically used (see Section 3.2.5). Potable water should be used for decontaminating drilling tools and mixing grout.

To assess the well construction materials' compatibility versus the subsurface environment and the pesticide of interest, manufacturers can provide data about the various well construction materials or samples can be acquired for laboratory analysis. Also, QC samples of each material can be collected during installation and preserved for laboratory analysis for potential sample bias, if necessary. In addition to well construction materials, the potable water used to clean drilling equipment and to prepare the grout and hydrate bentonite should also be collected for laboratory analysis (see Section 3.2.6).

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(1) Screen and riser. Well screen and riser pipes are available in a variety of materials, including steel, stainless steel, galvanized steel, fluorocarbon polymers (e.g., Teflon), fiberglass, polyvinyl chloride (PVC), and polypropylene.[5] Common screen and riser materials for groundwater monitoring wells are stainless steel and PVC. Materials are selected on the basis of their inertness relative to the groundwater conditions and the pesticide being investigated. Other usage concerns include strength, cost, durability, and workability. Although open-hole wells do not use a screen section, the riser pipe selection is important. Riser material must be compatible with subsurface conditions and must be strong enough to withstand the seating of the pipe in the bedrock and the bedrock drilling that follows its placement (Figure 1).

(2) Filter pack. Filter pack is the term used to describe the materials placed in the annular space between the screen and the formation (Figure 1). Aller *et al.*[4] and Driscoll[6] provide a discussion of the purpose and selection of filter pack materials. The filter pack serves a number of important mechanical functions. During well construction, it helps to keep the well screen centered in the borehole and acts to block the sealant from entering the well screen. The filter pack also improves hydraulic conductivity between the well screen and the formation, prevents formation materials from migrating to the well screen by acting as a filter, and helps support the screen and borehole.[6]

The most common filter pack material is quartz (silica). Quartz is relatively inert, readily available, and workable; therefore, it is preferred to replace formation materials removed from the borehole. The grain size of the filter material (i.e., sand or gravel) should be chosen based on the characteristics of the formation to be monitored and the slot size of the screen. Sand and gravel are available in various uniform sizes to accommodate different monitoring environments.

Unconsolidated or weakly consolidated sediments sometimes collapse around the well screen before the filter pack can be installed. This phenomenon is called 'formation collapse'. Formation collapse can occur as a result of the inherently unstable nature of certain sediments or the disruptive nature of the drilling process. Formation collapse is most common below the water table. Although steps can be taken to minimize the amount of collapse, it may not be entirely preventable. The groundwater monitoring plan may need to accept natural formation material as the filter pack for some or all of the screen section. Well development activities (see Section 2.1.6) can be designed to maximize the effectiveness of the formation collapse materials as a filter pack.

For some subsurface conditions, a geotextile (a woven fabric) can be wrapped around the well screen to provide the effect of the filter pack. The geotextile permits the passage of groundwater but is designed to prevent the passage of fine-grained material. Geotextiles can also be used in combination with a conventional filter pack. Similarly to a filter pack, the geotextile should be inert in relation to the pesticide being tested and be compatible with the surrounding subsurface conditions.

(3) Well seals. The annular space between ground surface and the top of the filter pack needs to be sealed. The seal prevents vertical migration of groundwater between different formations in the vicinity of the seal, prevents the infiltration of surface water or contaminants, prevents the upward migration of groundwater under confining conditions, and isolates the screened interval to a discrete portion of the aquifer.[2] The process of sealing the well to the surface is called grouting, and the sealant is called grout. To install the grout, the annular space is filled with a low-permeability material capable of adhering to the well casing and the formation materials (Figure 1). It is installed as a slurry to enhance its ability to fill gaps or voids in the borehole. In groundwater monitoring, well seals are commonly made of potable water mixed with either cement (neat cement grout), bentonite (bentonite grout) or a combination of the two (bentonite cement grout) materials.

Grout made of Portland cement and water is called neat cement.[1,4–6] The Portland cement and water are mixed together above ground into a slurry. The slurry should be thin enough to be circulated through a pump. The ratio of water to cement is limited because an excessive amount of water may cause the cement grout to shrink excessively and be weakened.[4] The curing process for neat cement is exothermic and, in boreholes with large annular spaces, the amount of heat generated may affect some casing materials (e.g., PVC, fluorocarbon polymers). Therefore, the installer should consult the manufacturer's specifications for temperature limits of the selected casing materials.

Bentonite is the name for a hydrous aluminum silicate comprised principally of the clay mineral montmorillonite, notable for its ability to swell in water and to form a very low-permeability seal.[4] It is available as powder, granule (chip), or pellets. Powder and granule sizes are produced by processing after mining. Bentonite powder

that has been compressed into nearly uniform spherical shapes and sizes is called pellets. Bentonite powder is commonly mixed with water to form slurry before being installed. Pellets and chips are installed dry and hydrated after installation.[4]

Bentonite products are most commonly installed in combination as pellets and slurry. The pellets are placed on top of the filter pack up to 1.0-m thick. If not below the water table, the pellets need to be thoroughly hydrated with potable water. Upon swelling, the pellets seal the casing to the borehole wall and act to prevent overlying liquid grout from migrating through the filter pack and entering the well screen.[7] Following its hydration, a bentonite grout is added to the borehole to complete the well seal.

As neat cement cures, it shrinks, and cracks may develop.[8] To reduce the amount of shrinkage, powdered bentonite can be added to the cement slurry. This grout is called bentonite cement grout. The curing process for bentonite cement grout is also exothermic and may affect some casing materials as discussed above.

Determining which grout to use is dependent on the goals of the monitoring plan and the characteristics of the site. Consideration must be given to the potential effect of the grout material on groundwater quality. Cement grout is highly alkaline and may affect groundwater pH.[4] Bentonite grout has a high cation-exchange capacity and may add or remove cations from groundwater. Aller *et al*.[4] and Lapham *et al*.[8] summarize the concerns of using bentonite and cement grouts. Considerations must also be given to whether the grout would be exposed to moving water or be needed to provide structural strength to the well. When exposed to moving water (e.g., high-permeability glacial gravel deposits with high-velocity groundwater), a neat cement grout, which hardens, may be preferred over a bentonite grout, which does not harden.[4] Neat cement grout may also be preferred for sealing open-hole well casings to the bedrock and overlying formation materials because drilling tools need to operate within the casing after the grout has cured. Because it hardens upon curing, neat cement grout would hold the casing more securely than bentonite grout.

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2.1.5 Well construction methods

Screened wells are constructed by assembling selected lengths of the screen and riser piping. Screen and riser sections can be purchased in a number of standard lengths to accommodate most groundwater monitoring plans. The sections are available with flush-joint threads, which eases both construction and subsequent use by eliminating the need for adhesives or couplings. Adhesives are not desirable since they might be reactive with water or the analytes being monitored.

To install screened wells, the drilling equipment must include a retractable casing. Hollow-stem auger flights provide this convenience (i.e., the inner-bore of the auger is hollow). Other drilling methods utilize temporary casing of a larger diameter (than the well materials) to maintain an open borehole. The augers or temporary casing serve to prevent premature collapse of the surrounding formation materials.

To install the screen and riser piping, the drilling equipment extends the borehole to the approximate desired depth of the bottom of the screen. Short sections of screen and/or riser pipe can be assembled above ground. A cap or plug (also with flush joint threads) is commonly added to the bottom of the lowest section to prevent the entry of formation materials. Precautions should be taken to keep the sections free of

contaminants. Personnel handling the well materials can prevent contamination of the well components by wearing clean disposable gloves. Handling long sections above ground may compromise the seal that the joints achieve when initially connected. Only short sections are assembled and handled above ground because of flexing. Once a safe number of screen sections or screen and riser sections have been assembled (or partially assembled), the casing is lowered into the borehole maintained by the retractable casing.

The screened section is lowered, with the bottom cap in place, into the retractable casing. If additional screen or riser-pipe sections need to be connected, the well sections are temporarily held at the top of the retractable casing, additional sections attached and the entire assembly is lowered until the screen is at the desired depth in the well borehole. A temporary cap or plug is applied to the top of the riser pipe to prevent debris from entering the well while construction continues. Once the well assembly has been lowered to the bottom of the well borehole, a clean measuring tape with a weighted bottom is inserted into the annular space and lowered to the bottom. The measuring tape is used to determine the amount of formation collapse that may have entered the bottom of the temporary casing and to monitor the installation of the filter pack to the appropriate height. For shallow wells, the filter pack can be poured into the retractable casing from above ground while the retractable casing is removed. Once the filter pack installation has begun, it should continue without stopping or be completed as quickly as possible to limit the segregation of large and small grain sizes caused by falling through water. Deep wells should have filter packs installed with a tremie pipe. A tremie pipe is a pipe or set of connected pipes that extend down the annular space to the target depth for well material placement. The filter pack may be poured into the tremie pipe while dry or with the addition of potable water (a sand slurry) to facilitate its passage through the tremie pipe.

Once the filter pack has been installed to approximately 0.6 m above the top slot of the screened section, a second filter pack of a very fine grain size may be installed (Figure 1). This secondary filter pack prevents the penetration of the grout through the primary filter pack to the well screen and is especially useful if the primary filter pack is composed of large diameter materials (e.g., gravel sizes). The thickness of the secondary filter pack is related to its grain size and to the penetration capability of the grout, the depth of the borehole, the grouting plan for the annular space, and the design limits on the length of the gravel pack. An alternative to using a secondary filter pack would be to extend the primary filter pack to the thickness achieved by the addition of the secondary filter pack.

The two most common methods to install grout are to pump the grout through tremie pipe or to pour the grout from the ground surface. The bottom of the tremie pipe discharge is usually capped to prevent the grout from being jetted through the filter pack materials and slots are cut in the sides of the tremie pipe at the bottom to allow the grout to discharge into the borehole. The sealant (neat cement grout, bentonite grout, or bentonite cement grout) is then pumped to the bottom of the remaining borehole through this pipe. The sealant is continuously pumped out of the tremie pipe into the base of the borehole and allowed to rise toward the ground surface. As the level of the grout rises, the retractable casing is lifted to keep it slightly above the grout level. By this method, the sealant can displace air and water and fill voids. Once the sealant is installed to the design height, the pumping stops and the

tremie pipe is removed from the borehole and the grout allowed to cure. Under some conditions, the retractable casing can be filled with grout before being removed.

If bentonite pellets or chips are to be used instead of grout, the bentonite pellets can be placed in shallow wells (within 15.0 m of ground surface)[8] by pouring them through the annular space from above in much the same manner as the filter pack is placed. If this installation procedure is chosen, sufficient potable water must be added in conjunction with the bentonite to hydrate the individual pieces. Hydration causes the pieces to swell and form a highly impermeable seal.

Installing monitoring well seals in gravelly deposits can be accomplished with solid bentonite products. A grout may migrate far beyond the well boring due to the pressure head developed in the retractable casing and high permeability of a gravel deposit. To prevent this migration, bentonite pellets can be dropped from above (or through a tremie pipe) and hydrated.

Once the grout materials have cured (dependent on grout composition and site conditions, but commonly approximately 24 h), a locking protective cover is installed in concrete over the top of the riser pipe. A concrete pad is commonly installed around the protective cover with its upper surface sloped away from the cover to shed precipitation and/or surface water (Figure 1).

2.1.6 Well development

Well development refers to the process of removing drilling fluids and fine-grained materials from near the well screen. Their removal helps the subsequent collection of nonturbid samples and improves the hydraulic connection between the well and the aquifer.

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Well development activities are usually a combination of vigorous agitation (surging water in the well, jetting potable water, injecting compressed air, etc.) of the groundwater, formation materials, and, if used, drilling fluids remaining in the well, combined with pumping. Agitation serves to keep those particles in suspension until they can be removed by pumping. It also attempts to dislodge and suspend drilling fluids and particles which have become lodged in pore spaces, moving them into the well, where they can be removed by pumping.

Another aspect of well development is the removal of drilling fluids. The discussion above focuses on drilling fluids that remain in the well proper. Some drilling fluids can migrate from the immediate area surrounding the borehole, out into the formation. For groundwater samples to be representative, these drilling fluids need to be removed. Monitoring the volume of the drilling fluid at the start of drilling and adding on supplemental fluids (volume) due to loss into the formation or deepening of the borehole is important. In this way, at the completion of well installation, an estimate of the total amount of fluids lost to the formation can be made. Development activity can then be directed to recovering the lost volume. Some synthetic drilling fluids are designed to degrade when additives are mixed with the drilling fluid during development. This greatly simplifies the removal process.

Pumping during well development performs two important functions. First, pumping removes the materials from the borehole left behind by drilling. Second, as the water in the well is removed, groundwater flow velocity from the surrounding formation increases when it reaches the higher permeability filter pack around the well

screen and may carry fine-grained materials into the well. This further improves the hydraulic connection of the screen to the formation. Raising and lowering the pump intake throughout the saturated portion of the well facilitates development of the entire screened interval. In this way, natural permeability from throughout the saturated portion of the formation may be improved. Similarly, the device chosen to agitate the water in the well should be applied throughout the saturated interval to develop the well fully.

If the size of the well permits, it is best to have the pump intake in the well and operating while surging is under way. This prevents particles from moving to another part of the well. Surging devices need to be selected on the basis of the characteristics of the well formation. The devices should be able to move the water vigorously enough to develop the well yet not so vigorously that it compounds the damage done to the formation by drilling or causes damage to the well screen.

2.2 Water supply wells

Groundwater monitoring wells are designed to collect discrete information about selected aspects of the subsurface. This information usually includes the ability to collect samples for laboratory analysis, determine the elevation of the water table and test the permeability of the aquifer. Water supply wells are designed to provide large volumes of groundwater over long periods of time (sometimes continuously). Some examples are wells for domestic (homeowner), irrigation, municipal, and industrial use.

There are many considerations to take into account when selecting supply wells for use as a groundwater monitoring point. Some of these considerations are the same as for constructing and sampling a monitoring well. There are additional concerns because of production volume associated with supply wells. Perhaps the most important consideration is well depth and screen length. As well screen length and depth increase, the portion of an aquifer from which sampling water may be withdrawn increases. This results in the collection of a sample that may not be representative of the water body under investigation. Determining whether water supply well samples are suitable for the purposes of the groundwater monitoring plan will depend on the goals of the plan and particular well under consideration. For a thorough description of criteria to consider in selecting supply wells for monitoring wells, the reader is referred to Lapham *et al*.[8]

2.3 Other groundwater sources

Springs, seeps, and drain tiles may be considered suitable groundwater sampling points. Springs and seeps are points on the land surface where groundwater discharges. They differ from each other in that a spring usually produces a larger volume of water per unit area than a seep. Springs may form the headwater source for small streams, whereas seeps, although able to wet relatively large areas, do not tend to generate enough groundwater to produce significant flow. Drain tiles are buried pipes or tubes that have been installed to remove shallow groundwater from an area. These tiles or tubes are typically buried 1–2 m below the ground surface.[9] Access to their

discharge points can provide groundwater samples. Depending on the size of the area drained, the tile discharge can provide information about a discrete area (if small) or nondiscrete area (if large). These groundwater sources are typically not sufficient in number, volume, persistence, or location to provide for an entire groundwater monitoring plan. However, they can be integrated into a plan as supplemental sampling points.

3 Groundwater sampling procedures

3.1 Pesticides of interest

The design of sampling procedures begins with the assemblage of physical and chemical characteristics of the pesticide of interest (e.g., volatility, density, photochemical conversion, half-life, solubility, temperature stability). It is important to establish the relationship of the study compounds of interest to the sampling procedures implemented as part of a monitoring plan. Specific sub-procedures may be necessary as part of the sampling design if multiple pesticides are being investigated (e.g., volatile vs nonvolatile compounds).

3.2 Sample collection techniques

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The goal of the sampling procedure is to establish methods that can be used consistently to collect representative groundwater samples. Different procedures are required to sample monitoring wells and water supply wells. Prior to the collection of groundwater samples, stagnant water must be removed from the well (and in the case of a water supply well, any water holding tank if there is no sampling port available prior to the tank). Stagnant water in the well is not representative of the aquifer conditions because the groundwater may have reacted with the well casing materials or undergone chemical changes with the atmosphere in the well. Field methods for water source purging and sampling are provided below.

3.2.1 Purging of the water source

The basic procedure for purging wells is to evacuate from a minimum of three to a maximum of five well volumes of water while monitoring specific groundwater parameters (e.g., pH, specific conductance, temperature, dissolved oxygen, turbidity). Once the minimum number of well volumes has been removed and groundwater parameters have stabilized, stagnant water can be replaced by groundwater from the aquifer and water samples can be collected. Section 3.2.2 provides a discussion of groundwater parameter measurement and stabilization criteria.

The well volume needs to be determined before beginning the purging process. Record the following information to calculate the well volume: the depth to water (measured using a clean, electronic water-level measuring device; see Section 3.2.5), the total depth of the well (determined using a clean well sounding device or obtained from well construction logs), and the inside diameter of the well casing. Document

all field measurements and calculations (Figure 2). The volume of water in a well is calculated by using Equation (1) for British units[10] or Equation (2) for Système Internationale (SI) units:

$$V = 0.041\, d^2 h \tag{1}$$

where $h =$ water column height in feet, $d =$ diameter of the well in inches, and

WATERBORNE ENVIRONMENTAL, INC.

Monitoring Well Purging and Sampling Form: MW___ and MW___
Event_____

Study: Prospective Ground-Water Monitoring Study
Study Protocol No.: 12345 WEI Project No.: 678.90

Please note the following: Reference SOP for purging and sampling is WEI-8DD. Dedicated bladder pumps are used for purging and sampling. Sample IDs are listed on the Chain-of-Custody form.

Equipment IDs:_____

Sampling Personnel:_____

Calculate Purge Volume For Well ID No.: MW_____

(1) Total well depth (m)	[*]	_____	Purge Start Time:_____
(2) Depth to water (m)		_____	Sampling Time:_____
(3) Water column (m)	[(1) - (2)]	_____	Duplicate Sample Collected:
(4) Well volume (L)	[(3) x 2.0]	_____	Yes_____ No _____

Time	Cumulative Vol. (L)	Temp. (°C)	pH	Cond. (µS)	Dissolved Oxygen (mg/L)	Turbidity (NTU)

*From well construction record

Calculate Purge Volume For Well ID No.: MW_____

(1) Total well depth (m)	[*]	_____	Purge Start Time:_____
(2) Depth to water (m)		_____	Sampling Time:_____
(3) Water column (m)	[(1) - (2)]	_____	Duplicate Sample Collected:
(4) Well volume (L)	[(3) x 2.0]	_____	Yes_____ No _____

Time	Cumulative Vol. (L)	Temp. (°C)	pH	Cond. (µS)	Dissolved Oxygen (mg/L)	Turbidity (NTU)

*From well construction record

Additional Comments:_____

Recorded by:_____ Date:_____

Figure 2 Example of a monitoring well purging and sampling form

V = volume of water in gallons;

$$V = 0.001\pi r^2 h \tag{2}$$

where $h =$ water column height in centimeters, $r =$ radius of the well in centimeters, $V =$ volume of water in liters, and $\pi = 3.14159$.

The volume of water to be purged from the well must be calculated. Methods for determining purge volume include the use of a flow meter on the discharge line, the discharging of purge water into a calibrated container, and/or purging at a known flow rate for a specific period of time.

Groundwater is removed from the well by pumping or bailing. The rate of discharge from a well during the purging process is dependent on the hydraulic properties of the aquifer and the method used to withdraw groundwater from the well. It is preferable to purge the well slowly while striking a balance between the time required to purge the well and the spacing between groundwater parameter measurements. It is best to use the same device for purging and sampling. Bailing is not a preferred method because it causes turbidity in the well and may cause volatilization of volatile organic compounds.[7] If a bailer (fluorocarbon polymer or stainless steel) must be used, it should be slowly lowered into the top of the water column, allowed to fill, and then slowly removed. Care must be taken when using a bailer that the lanyard and the bailer do not come in contact with sources of contamination outside the well (e.g., the ground or other equipment). Equipment decontamination (or disposal in the case of the lanyard) should occur between wells and/or between uses if the bailer is not dedicated to a specific well.

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Several pump types are available for purging wells and include submersible positive displacement (e.g., gear-driven, bladder, helical rotor, piston, centrifugal) and gas contact (e.g., gas lift or gas driven) pumps.[3,5] A peristaltic pump may be used for shallow groundwater sampling [less than 7.6 m below ground surface (bgs)].[11] All equipment should be properly decontaminated prior to use. Existing equipment is used for monitoring wells with dedicated pumps or water supply wells with plumbing in place during the purging and sampling process.

Methods to remove stagnant groundwater from a well are dependent on the design of the well and whether the well has a dedicated or a permanent pump in place. For wells that have a screened or open-hole interval completely below the water table and do not have dedicated pumps or plumbing in place, stagnant water is removed from the well by gradually lowering the pump or pump tubing into the water column.[7] Placement of the pump in the top of the water column or above the well screen may facilitate water flow from the entire screened interval up to the pump; however, more permeable zones of the aquifer may contribute more recharge to the well than other less permeable zones. The pumping rate during purging should not markedly lower the water level or lower the water level into the open screened interval.[11] This method is preferred to running the pump in the screened interval because the water withdrawn from the well may be derived from the adjacent aquifer section and not from the entire water column. If the pump is removed after purging is complete and a bailer is used to collect a sample from the top of the water column, the sample may not be representative of the aquifer because the entire water column was not purged.[7]

In cases where wells have a screened or open-hole interval partially submerged below the water table and a pump is going to be used for both purging and sampling, the pump should be placed at the midpoint between the top of the water table and the bottom of the screen.[10] If different equipment is going to be used for purging and sampling of the well, the pump should be placed at the top of the water column.[10]

For wells that have dedicated equipment or have plumbing in place, standard purging procedures (e.g., well volume and parameter criteria) should be followed for evacuating stagnant groundwater. It may be difficult to purge all stagnant water from wells in aquifers with a high hydraulic conductivity where the pump is placed in the screened section or open-hole section of the well.

In some cases, the geological formation has a very low yield and the well may purge dry. Purging a well dry should be avoided, if possible, by balancing the groundwater removal rate with the rate of recharge to the well. When a well is purged dry, groundwater may cascade into the well, altering the water chemistry or increasing its turbidity.[7] If a well purges dry, no further purging of the well is necessary. Attempt to purge at least two volumes of water equal to the volume of the purging system (e.g., pump and tubing).[10] The water level then should be allowed to recover and groundwater parameters (e.g., pH, specific conductance, temperature, dissolved oxygen, turbidity) should be measured when the groundwater samples are collected.

Low-flow purging is an alternative method for purging a well prior to sampling and has advantages over standard purging methods because the procedure reduces the volume of water discharged from a well, minimizes the stress on the aquifer and potential aeration of the groundwater sample, and minimizes the suspension of sediments in the well and from the formation.[7,12] This method of purging is accomplished by setting the pump at a low discharge rate (200–500 mL min^{-1}) that will not cause excessive drawdown of the water level in the well. Water levels in the well are monitored until the discharge rate produces only minor drawdown (9 cm or less).[12] Purging is complete when the groundwater parameters stabilize.

(1) Purging of continuously or intermittently pumping water supply wells. The sampling procedures for water supply wells (i.e., domestic water wells, irrigation wells, public/industrial wells) follow the same criteria as for monitoring wells (i.e., purge well volumes and measure groundwater parameters for stabilization).[7] The volume to be purged from a water supply well prior to sample collection depends on the total volume of water in the well and the pumping/plumbing system up to the sampling point and whether the well pump is running continuously or intermittently. Sampling downstream of a storage/pressure tank should be avoided. If storage/pressure tanks are present, an adequate volume must be purged to totally exchange the volume of water in the tank. All samples must be collected from the closest spigot to the wellhead, with all screens or aerators removed, and with the flow rate reduced. Also, samples should represent raw groundwater and not be collected downstream of a water treatment system. If necessary, a sampling valve may need to be installed upstream of any treatment or storage equipment.

If the pump runs continuously and the sample can be collected upstream of a storage/pressure tank, no purging is required, other than opening a sampling valve and allowing it to flush for a few minutes. All spigots or valves on the system located downstream of the storage tank should be opened to prevent backflow from the

tank.[7] If the pump runs continuously and a storage/pressure tank is located upstream of the sampling location, locate a sampling valve closest to the tank. Groundwater parameters are measured prior to the collection of groundwater samples.

If the pump runs intermittently, it is necessary to determine the volume to be purged, including that of the delivery piping and storage/pressure tanks that may be located upstream of the sampling location. The pump should then run continuously until the required volume has been purged. The pumping rate needs to be determined during purging (from all open valves). Measurement of groundwater parameters should be monitored at intervals during the purging process and at the time of sample collection. The pumping rate used to collect final groundwater parameters should be used for sample collection.[11]

When the well depth or diameter is unknown or a water-level measurement is not possible, purging should be carried out by pumping the well for a pre-determined period of time (e.g., at least 30 min).[7] Measurement of groundwater parameters should be monitored at intervals during the purging process and at the time of sample collection (Figure 3).

(2) Disposal of purge water. Follow regulations of the local jurisdiction for disposal of purge water. At a minimum, dispose of purge water a sufficient distance from the well so as not to affect the groundwater quality in the well or compromise the objectives of the monitoring study by allowing concentrated recharge.

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3.2.2 Monitoring of groundwater parameters during purging

Groundwater parameter measurements (e.g., pH, specific conductance, temperature, dissolved oxygen, turbidity) are collected at intervals during the purging process. Prior to purging, all field equipment must be calibrated and each field instrument must have an equipment maintenance log to document calibrations and maintenance. Equipment calibration should occur at the beginning of the sampling day. The equipment should be checked against standards at a midpoint during the sampling day, and either checked or recalibrated at the completion of sampling to ensure proper equipment operation. Back-up equipment should be available on-site in case of malfunction of the primary instruments. The parameter measurements are recorded from instruments installed in an airtight flow-through cell (i.e., a chamber where purge water from the well passes through) or from instruments placed in a container with a grab sample of fresh purge water. Document all measurements on field forms and note any deviations from established procedures (Figures 2 and 3).

As discussed before, groundwater samples can be collected when a sufficient volume of water has been removed from the well (e.g., three to five well volumes) and groundwater parameters have stabilized. If parameters have not stabilized after five well volumes have been removed, then the well may be sampled (acceptance of sampling following the fifth purge volume is dependent on the study objectives).[7,10] Table 1 summarizes the criteria used for establishing the stability of groundwater parameters.[7,10,11] The time intervals between the parameter measurements depend on the well characteristics and the hydraulic properties of the aquifer and must be sufficiently spaced to provide results representative of aquifer properties.[13]

WATERBORNE ENVIRONMENTAL, INC.

Irrigation Well Purging and Sampling Form
Event_____

Study: Prospective Ground-Water Monitoring Study
Study Protocol No.: 12345 WEI Project No.: 678.90

Please note the following: Reference SOP for purging and sampling is WEI-8OO. Sample IDs are listed on the Chain-of-Custody form.

Equipment IDs:_____

Sampling Personnel:_____

Purging Data For Irrigation Well:

Purge Start Time:_____
Sampling Time:_____
Duplicate Sample Collected:
Yes _____ No _____

Time	Cumulative Vol. (L)	Temp. (°C)	pH	Cond. (µS)	Dissolved Oxygen (mg/L)	Turbidity (NTU)
_____	_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____	_____

Irrigation Flow Meter (FM-1)

Flow Reading (L/m):_____ Time of Measurement:_____(am/pm)
Volume Reading (Liters):_____

Additional Comments:_____
_____
_____
_____
_____

Recorded by:_____ Date:_____

Figure 3 Example of a water supply well purging and sampling form

3.2.3 Sample collection

Sample collection and processing can take place once the purging process is complete. The purging process establishes that the well is producing groundwater representative of aquifer conditions. The supplies and equipment used for sample collection should not alter the sample or cause cross-contamination (positive or negative bias). All personnel conducting sample collection should be properly trained. It is of the utmost importance to maintain a clean work area and that sample handling procedures

Table 1 Stabilization criteria for groundwater parameter measurements

Field measurement	USGS[a]	USEPA[b]	FL DEP[c]
pH	±0.1 standard units	±0.1 standard units	±0.2 standard units
Temperature (°C)	±0.2 °C (thermistor thermometer) ±0.5 °C (liquid-in-glass thermometer)	Constant for 3 consecutive readings	±0.2 °C
Specific electrical conductance ($\mu S\,cm^{-1}$ at 25 °C)	±5% when ≤100 $\mu S\,cm^{-1}$ ±3% when >100 $\mu S\,cm^{-1}$	±10%	±5%
Dissolved oxygen concentration ($mg\,L^{-1}$)	±0.3 $mg\,L^{-1}$	Not applicable	<20% of saturation at field-measured temperature; if primary criteria are not achievable use ±0.2 $mg\,L^{-1}$ or 10%, whichever is greater
Turbidity (nephelometric turbidity units, NTU)	±10% for NTU <100	<10 NTU	<20 NTU; if primary criteria are not achievable use ±5 NTU or 10%, whichever is greater

[a] USGS = United States Geological Survey. Allowable variation between five or more sequential field measurement values taken 3–5 min apart. For wells with large purge volumes, take field measurements approximately 15 min apart.[11]
[b] USEPA = United States Environmental Protection Agency. No specified time interval between field measurements. If the purge volume is small, collect measurements with enough frequency to evaluate stability. If the purge volume is large, take field measurements approximately 15 min apart.[7]
[c] FLDEP = Florida Department of Environmental Protection. Allowable variation between two consecutive readings taken at least 2–3 min apart following the collection of one well volume.[10]

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are employed to eliminate the potential for cross-contamination. Sample collection should always proceed from no or low-concentration monitoring locations to high-concentration monitoring locations.

(1) Supplies and equipment for sample collection and handling. The supplies and equipment utilized to collect groundwater samples are dependent on the objectives of the monitoring program. Laboratory studies should be conducted in advance of designing the monitoring procedures to determine if sampling supplies (e.g., sample bottles) and equipment (e.g., well materials, pump materials) will contribute to or remove from the potential concentration of the compound of interest in the water sample.[14] All field supplies and equipment should also be able to withstand cleaning solutions used for decontamination.[14]

Unless laboratory studies on material compatibility establish otherwise, it is recommended that equipment used to collect groundwater samples for pesticide analysis be constructed of metal, fluorocarbon polymer, or glass.[11] However, for a water-supply well, inert well, pump, and plumbing materials are not likely to have been installed for all components. In this case, in-place well, pump type, and plumbing materials should be documented.

The type of sample containers and the volume of sample required for analysis are study specific. Laboratory-cleaned amber-glass sample bottles (1–3.8-L) are required for various types of pesticides and 40-mL amber-glass vials for volatile organic compound (VOC) pesticide samples.[7,15] Plastic bottle caps should have a fluorocarbon polymer liner. It is recommended that glass bottles have an exterior plastic coating when sample storage conditions are below freezing ($<0\,^{\circ}C$) to minimize sample bottle failure. Amber-colored high-density polyethylene (HDPE) bottles may be determined to be suitable through laboratory investigations. HDPE bottles are advantageous because the potential for sample bottle failure during sample handling and shipment is less likely than when glass is used.

To prevent cross-contamination, great care should be taken when handling sampling equipment or sampling containers. Work areas should be covered with clean disposable materials such as aluminum foil or plastic sheeting. Disposable, powderless latex or nitrile gloves should be used for sample collection and sample processing.[14] Gloves should be changed frequently. Attempt to avoid temperature extremes between groundwater samples and sampling equipment, avoid direct sunlight, protect sampling equipment and bottles from contamination sources (i.e., dust, other contaminated equipment), and avoid touching potential sources of contamination (e.g., treated soil).

(2) Sample processing. After purging, the collection of samples from the water source can be completed. It is preferred that the purge equipment be used to sample the well. Bladder pumps and other submersible pumps constructed of suitable materials are preferred for sample collection because they deliver a sample directly from the well to the sample container. A peristaltic pump can be used, but an intermediate sample container may be necessary because the flexible tubing in the pump head may not be inert and could cause sample bias. Although not preferred, a fluorocarbon polymer or stainless-steel bailer may be used to collect samples (see Section 3.2.1). Sampling equipment should be rinsed with well water prior to use if it is not used for purging.

The flow rate from the well during sampling should be similar to that under which purging was conducted and as close to the actual groundwater flow rate as possible.[3] The flow should not be interrupted during the collection of samples and should not exceed 500 mL min^{-1} for bottles 250-mL or larger or 150 mL min^{-1} for 40-mL VOC vials.[11] Samples for VOC analysis should be collected before other pesticide samples because the purging process might result in diminished VOC concentrations.[14] If samples need to be filtered (e.g., because of high turbidity), then an unfiltered raw water sample and a filtered sample should be collected.[7] The unfiltered raw water sample is collected prior to the filtered sample.[14] Sample bottles should not be field rinsed.[14]

The chemical preservation of a sample is dependent on the chemistry of the groundwater (e.g., pH) and on the chemical characteristics of the pesticide being studied. Preservatives can be added to the sample containers in the field or prepared in advance at the laboratory. To determine the need for a chemical preservative in the field (i.e., pH analyses), test the groundwater collected during the purging process and not the sample collected for analysis.

Label sample bottles prior to sample collection and always double check the label prior to collecting a sample. All sample labels should include the study number

and location, the sample matrix or type, the sample location, the sample depth (if applicable), the target sampling interval, and a unique identification number. The sample date and initials of sampling personnel may be written on the label at the time of collection, prior to securing with clear packing tape. Never set the sample cap down on an unclean surface or allow the sample bottle lip to come in contact with the sample discharge line, sampling valve, etc. Fill the sample bottle to the neck when the sample is going to be stored unfrozen and approximately 75% full when the sample is going to be frozen. The additional unfilled bottle space allows for sample expansion when it freezes.

Special attention needs to be given to collecting samples for VOC analysis. The VOC vial should be filled slowly until a convex meniscus is present above the sample vial lip. Carefully cap the sample vial, invert and tap to check for bubbles. If bubbles are present, then a new sample vial should be filled.

Sample bottle caps may be secured with electrical tape following collection (not VOC samples). Glass sample bottles should be wrapped in a protective cushioning material (e.g., bubble wrap) to provide protection against breakage during storage and shipment. Make sure the bottoms of the bottles are also cushioned. All sample bottles should be placed in labeled zip-press closure bags.

3.2.4 Sample storage and shipment

Sample storage conditions are largely based on the stability of the compound being investigated and the time interval between sample collection and laboratory analysis. Samples are placed immediately in storage conditions designated for the study following their collection (i.e., chilled on wet or blue ice packs at $<4\,^{\circ}C$ but $>0\,^{\circ}C$, or frozen on dry-ice). Samples should be segregated in the field by sample location and by levels of expected concentration (e.g., high versus low) and stored in the correct labeled storage container. Decontaminate the storage containers between each use (see Section 3.2.5).

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Groundwater samples are transferred to the laboratory for analysis at the completion of field activities. The transfer of samples may be direct from field to the laboratory, via overnight carrier, or via ground delivery in a freezer truck. It is important to follow all requirements of the overnight carrier to ensure that no delay occurs with the sample transfer. Samples should be stored under prescribed conditions with temperature monitoring and documentation if samples cannot be shipped immediately following collection (e.g., no Saturday delivery, freezer truck not scheduled). Verify that all samples are accounted for, the sample labels are secure, that all samples are in good condition, and complete the chain-of-custody documentation (COC). The COC serves as a record of samples collected in the field and transferred to the laboratory, the sample preservation and storage conditions of the samples, and the required analyses for the samples (Figure 4). The original COC is placed in a zip-press closure bag and taped to the inside of the lid of the shipping container. A verified copy of the COC must be retained by the field member responsible for shipping the samples.

Samples should be chilled or frozen to maintain stability of the analytes during shipment. Usually samples are packed and shipped on wet ice or dry-ice or stored and shipped in a freezer truck. Use a waterproof plastic insulated shipping container (e.g., cooler) and double bag the ice when samples are shipped on wet ice. A large

WATERBORNE ENVIRONMENTAL, INC.
CHAIN OF CUSTODY RECORD

Study: Ground-Water Monitoring Study
Protocol No.: 12345
WEI Project No.: 678.90

Location: _____
Sampler Name: _____
Send Results To: Waterborne Environmental, Inc.
897-B Harrison Street, SE
Leesburg, Virginia 20175
Phone: 703.777.0005
Sampler's Signature: _____

Ship To: _____
_____
Analysis For: _____
Preservation: _____
Airbill No.: _____

Bill To: _____
Notes: _____
_____
_____

Sample I.D.	No. of Samples	Sample Date	Additional Comments/Condition upon Receipt at Lab	Laboratory I.D.

Relinquished By (Sign):	Date/Time:	Received By (Sign):	Date/Time:

Figure 4 Example of chain-of-custody form

heavy-duty plastic bag may be used to provide additional protection from water leakage. Make sure there is plenty of ice to preserve the samples during transport. Secure the drain plug in the closed position on the shipping container with duct tape, if present. Make sure sufficient packing materials are used to protect sample container integrity and any void space is filled in the shipping containers for all shipments. If warranted, special sample shipment containers may be manufactured to secure sample bottles in insulated foam with slots provided for blue ice packs (e.g., for a study with large numbers of samples). Samples should be chilled to $<4\,^{\circ}C$ prior to using this type of shipping container.

When preserving and shipping samples on dry-ice, sample containers should be enclosed in a cotton/nylon bag or packing materials (e.g., paper, cardboard) to prevent direct contact with the dry-ice. Typically, 13–23 kg of dry-ice are needed for a large shipping container (e.g., 45-L volume shipping container) to maintain freezing temperatures for overnight (express) shipping. Dry-ice should be handled carefully and the manufacturer's Material Safety Data Sheet should be followed for safety precautions. If a freezer truck is being used to deliver the samples to the laboratory, the

samples should be frozen prior to loading on the truck. Heavy-duty cardboard boxes or other insulated containers may be used to ship samples via this method.

3.2.5 Decontamination of sampling equipment

The goal of decontamination is to prevent contamination (positive bias) of groundwater samples by removing all pesticide residues and other potential interfering materials from sampling equipment.[7,15,16] Decontamination procedures must be documented. All sampling equipment that comes in contact with groundwater must be cleaned or disposed of between sampling events (e.g., water-level indicators, pumps and associated tubing, bailers, and sample processing and shipment equipment).

For pesticide monitoring studies, the general procedure for decontaminating field equipment includes a dilute wash with a solution of potable tap water and a non-phosphate or laboratory-grade detergent, a potable tap-water rinse, an analyte-free water rinse (e.g., deionized or distilled water), followed by a pesticide-grade solvent rinse (e.g., acetone, methanol, 2-propanol). Depending on study requirements, a final analyte-free water rinse may be required. Do not use a solvent rinse with noncompatible materials (e.g., plastics). It is important to make sure that all cleaning solutions have been rinsed from the equipment before use. Equipment quality control samples should be collected as necessary (see Section 3.2.6). All decontaminated equipment should be handled in a manner to prevent recontamination. Equipment should be dried following cleaning and stored in aluminum foil or protective plastic bags and labeled as 'cleaned' and with the date of cleaning. Proper disposal practices should be employed to dispose of contaminated wash solutions. Safety procedures must be in place when handling cleaning solutions.

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3.2.6 Field QC samples

Field QC sampling is a necessary component of a groundwater monitoring study and should be integrated into groundwater sampling procedures.[11,15] The purpose of collecting QC samples is to provide data for the evaluation of potential bias and the evaluation of measurement variability that may result from a combination of field and laboratory errors.[15] The QC data are used to verify the quality of the groundwater monitoring data. Results for QC samples are also used to determine suitable corrective actions in sampling procedures or used to refine the monitoring plan.

The following is a summary of QC sample types.[10,11,15] The three main types of QC samples are field blanks, replicates, and field fortified samples. Field blanks are QC samples to determine sample bias caused by contamination sources and include source solution or analyte-free water blanks, equipment blanks, trip blanks, and ambient blanks. A source solution or analyte-free water blank is collected to establish that the water being used for QC samples is free of the compounds of interest. This sample should be collected in a contaminant-free environment. Equipment blanks establish the adequacy of decontamination procedures and are collected by passing analyte-free water through field equipment following equipment decontamination. These blanks should be collected following the sampling of a monitoring point that is known to have some concentration of the compound of interest. Trip blanks are used to determine if samples are contaminated during sample storage and shipment.

Trip blanks are prepared by filling a sample container with analyte-free water and placing the sample in a field storage container prior to sample collection. Trip blanks are usually provided by the analytical laboratory. The trip blank remains with the water-quality samples until receipt at the laboratory. Trip blanks should be used when sampling for VOCs. Ambient blanks are collected to determine if samples can be contaminated through exposure to the environment during the collection process. This sample may be prepared by pouring analyte-free water into a sampling container and allowing it to be exposed to the atmosphere for the period of time a water-quality sample would be exposed.

Replicate samples are collected to evaluate the measurement variability of field and laboratory procedures. When sampling a water source, replicate samples (two or more) should be collected sequentially. Select wells for replicate sampling that are known to have a measurable concentration of the compound of interest.

Field-fortified samples are collected to evaluate sampling bias that may result from the sample matrix and/or field and laboratory procedures. Field-fortified samples are prepared by adding a solution of known concentration to a known volume of analyte-free water or groundwater. Fortification concentrations should represent the range of concentrations expected in groundwater samples. Control groundwater samples should be collected from representative control wells (in untreated areas) that do not contain the pesticide of interest. If measurable quantities of pesticides are present in fortified samples then a positive bias will occur. Nonfortified samples should always be collected when spikes are prepared. Lastly, blind spikes are used to test for bias of laboratory procedures. A blind spike is simply submitting a field spike sample to a laboratory, but withholding information on the concentration of the fortification sample from the laboratory.

The quantity of QC samples to be collected is dependent on the study design, but field blanks and field replicates should represent approximately 5–10% of the groundwater samples collected for the study. QC samples should be collected on the same day, using the same supplies and equipment, and be stored and shipped under the same conditions as the groundwater samples collected for pesticide analysis. Document all procedures, equipment, and reference chemicals used to generate the QC samples.

3.3 Sampling of other groundwater sources

Shallow groundwater discharge in the form of springs, seeps or shallow groundwater drainage from drain tiles in agricultural areas may be sampled as part of a groundwater monitoring study. As with the groundwater sampling procedures discussed in previous sections, the goal of sampling is to obtain a water sample that is representative of shallow groundwater quality.

The procedures for obtaining a water sample from a spring, seep, or drain tile are highly dependent on the construction or landscape position of the water source. Sample collection may proceed by sampling at the drain tile outflow point or at the spring discharge point, or may require the excavation of soil and temporary installation of a water collection reservoir and/or discharge pipe to permit sample collection. The sophistication of the instrumentation of the sampling point is a function of the monitoring

plan (i.e., number of sampling events). The materials used for the instrumentation should be compatible with the pesticides of interest. It is extremely important to be careful to prevent cross-contamination when working with shallow groundwater sources near agricultural fields because of the potential presence of pesticides in soil and in airborne dust. Procedures described above for groundwater sample processing, for sampling supplies, and for sample storage and shipment also apply to sampling of springs, seeps, and drain tiles.

Document all field observations on the appropriate forms. Written and photographic documentation of the sampling locations should record the design and materials used for construction of the sampling location, the procedures used to collect samples, and any potential sources of contamination that may bias the sample results.

4 Suction lysimeter installation and sampling procedures

Suction lysimeters are required for some field-scale groundwater monitoring studies to monitor the transport of compounds of interest through the unsaturated zone.[1] Unlike monitoring wells or water supply wells that sample water from the saturated zone, suction lysimeters sample water from the unsaturated zone. This section provides a summary of the installation and sampling procedures for pressure-vacuum suction lysimeters. A detailed discussion of unsaturated zone sampling devices is available elsewhere.[17]

Pressure-vacuum suction lysimeters are constructed of a reservoir section connected to a porous cup with vacuum/pressure and sample access lines (Figure 5). The lysimeter is operated by applying a vacuum to the lysimeter through the vacuum/pressure line. The pressure gradient created by the vacuum between the porous cup and the surrounding formation will cause soil water to flow into the lysimeter reservoir. The rate at which soil water collects depends on the hydraulic conductivity and matric potential of the soil and the strength of the vacuum created in the reservoir. The water collected in the reservoir is returned to the surface by applying pressure (e.g., pumping air or inert gas) to the reservoir through the vacuum/pressure line, which causes the water to discharge through the sample line for sample collection.

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The reservoir materials may be PVC, stainless steel, or a fluorocarbon polymer, and the porous cup may be constructed of ceramic, stainless steel, or fluorocarbon polymer. Ceramic cups have a smaller pore size, a greater bubble pressure (pressure under which the cup produces bubbles), and a greater operational suction range,[17] and are preferred to other porous cup materials. All materials used for the construction of the suction lysimeter should be tested in the laboratory to determine if any bias in the sample analysis will result from their use.

4.1 Preparation and installation

Lysimeter assembly and pre-installation testing should be conducted in a clean area, preferably indoors. Careful installation is necessary to ensure proper operation. The components of the lysimeters may need to be decontaminated prior to installation

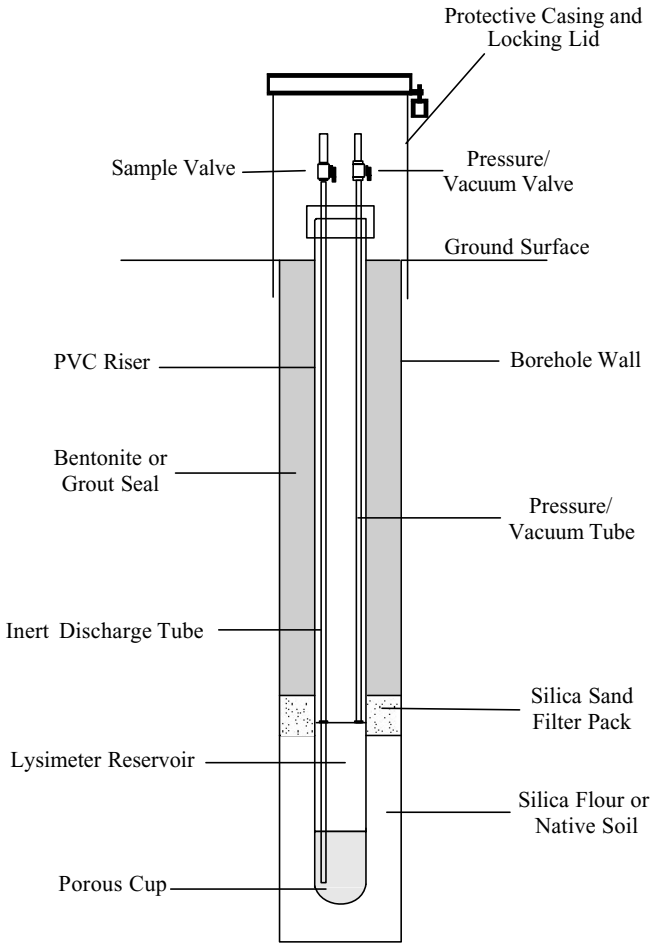


Figure 5 General construction of a suction lysimeter

(see Section 3.2.5). The manufacturer's instructions should be followed for decontamination of the porous cup.

Make sure that all fittings are secure when assembling lysimeters and that all lysimeters are tested before installation. Three pre-installation tests are recommended and include a pressure test for leaks and an even flow of bubbles from the cup by applying air or nitrogen gas under pressure through the vacuum/pressure line while submerging lysimeter and tubing connections in analyte-free water, a vacuum test for leaks using an airtight latex membrane secured with tape to seal the porous cup, and the placement of lysimeters in a container of analyte-free water under vacuum. The final testing procedure verifies proper tube assembly and also serves to pre-wet the porous cup.

The method to install a lysimeter is dependent on the placement depth. A hand auger may be adequate to advance a shallow borehole (<4.5 m), or a hollow-stem auger may be necessary to advance a deeper borehole. If a hollow-stem auger is used,

slough soil will be present in the bottom of the borehole and should be removed prior to installation.

A number of design and installation methods are possible.[18,19] The porous cup should be in intimate contact with the soil for soil moisture to move readily from the pores of the soil through to the pores of the cup and into the sampler. Silica flour (<200-mesh ground silica) or native soil can be used to create this 'bridge' between the soil and the porous cup. The homogeneity of the soil is a factor since the primary concern is that the porous cup is in tight, intimate contact. The silica flour slurry or native soil slurry is poured from the ground surface into the borehole or poured through a tremie pipe.

After the silica flour or soil mixture has set, add a layer of silica sand as a filter pack, then fill the remainder of the borehole with bentonite or a grout seal (Figure 5). Access lines may be run from the lysimeter reservoir through a protective PVC riser pipe to the land surface. A locking protective casing should be used to secure the above-grade lysimeter components. If the lysimeter is used immediately after installation, it is necessary to purge any water used to prepare the silica flour or soil slurry. Remove at least the water volume equivalent to approximately one third of that used to make slurry.

4.2 Lysimeter sampling

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The major difference between sampling suction lysimeters and groundwater sampling is that no purging of the lysimeter takes place and only a limited amount of soil water is available to be sampled. Typically, soil-water parameters (e.g., pH, temperature, electrical conductance) are not measured owing to the limited volume of soil water available during a sampling event. When residues are present in soil-water samples, those residues may produce a positive bias in soil-water samples from subsequent sampling events because the lysimeter reservoir cannot be purged or decontaminated.

The sampling of a suction lysimeter is initiated by applying a vacuum (approximately 40–50 cm of mercury) through the vacuum/pressure line with a hand pump or electric pump. The valve on the sampling line must be closed. A constant vacuum may be maintained on the lysimeter using an electric pump. The time required before collecting a sample from a lysimeter will depend on the method of vacuum application, the moisture content of the soil, and the soil type.

After sufficient time has been allowed to elapse (e.g., more than 2 h), open the sample and vacuum/pressure valves and allow the lysimeter to equilibrate with atmospheric conditions. Connect a pump or inert gas cylinder to the pressure/vacuum tube. By slowly pressurizing the lysimeter through the pressure/vacuum tubing, water collected in the lysimeter will begin to discharge through the sample tubing. Caution should be used when pressuring the reservoir so the sample is not forced back through the porous cup. Collect the sample as described for groundwater sample processing. Once all of the necessary sample volume has been collected, discharge the remaining water in the lysimeter into a calibrated container, record this volume and dispose of the water appropriately. Document all field observations on the appropriate forms (Figure 6). The types of sample supplies and the sample storage and shipment procedures discussed for groundwater sampling also apply for soil-water sampling.

WATERBORNE ENVIRONMENTAL, INC.

Lysimeter Sampling Form
Event_____

Study: Small-Scale Prospective Ground-Water Monitoring Study
Study Protocol No.: 12345 WEI Project No.: 678.90

Reference SOP: WEI-8N Sampling Personnel:_____

Vacuum Application

Time	Pump ID	Affected Lysimeters (circle)
1. _____ (am/pm)	_____	Full Cluster; L__-3; L__-6; L__-9; L__-12
2. _____ (am/pm)	_____	Full Cluster; L__-3; L__-6; L__-9; L__-12

Sample Time		Sampled Lysimeters (circle)
1. _____ (am/pm)	_____	Full Cluster; L__-3; L__-6; L__-9; L__-12
2. _____ (am/pm)	_____	Full Cluster; L__-3; L__-6; L__-9; L__-12

Lysimeter ID: L_____	Pump Gage	Lys. Gage*	Estimated volume collected for:
Vacuum At Start 1 (cm Hg):	_____	_____	Lab 1: (ml) _____
**Vacuum At Start 2 (cm Hg):	_____	_____	Lab 2: (ml) _____
Vacuum At End 1 (cm Hg):	_____	_____	Volume Remaining (ml): _____
Vacuum At End 2 (cm Hg):	_____	_____	Estimate Total Volume Purged (ml): _____

Lysimeter ID: L_____			Estimated volume collected for:
Vacuum At Start 1 (cm Hg):	_____	_____	Lab 1: (ml) _____
**Vacuum At Start 2 (cm Hg):	_____	_____	Lab 2: (ml) _____
Vacuum At End 1 (cm Hg):	_____	_____	Volume Remaining (ml): _____
Vacuum At End 2 (cm Hg):	_____	_____	Estimate Total Volume Purged (ml): _____

Lysimeter ID: L_____			Estimated volume collected for:
Vacuum At Start 1 (cm Hg):	_____	_____	Lab 1: (ml) _____
**Vacuum At Start 2 (cm Hg):	_____	_____	Lab 2: (ml) _____
Vacuum At End 1 (cm Hg):	_____	_____	Volume Remaining (ml): _____
Vacuum At End 2 (cm Hg):	_____	_____	Estimate Total Volume Purged (ml): _____

Lysimeter ID: L_____			Estimated volume collected for:
Vacuum At Start 1 (cm Hg):	_____	_____	Lab 1: (ml) _____
**Vacuum At Start 2 (cm Hg):	_____	_____	Lab 2: (ml) _____
Vacuum At End 1 (cm Hg):	_____	_____	Volume Remaining (ml): _____
Vacuum At End 2 (cm Hg):	_____	_____	Estimate Total Volume Purged (ml): _____

* Record lysimeter gage reading, if present.
**A vacuum may be applied to a lysimeter more than one time. The previous vacuum is lost when an additional vacuum is applied.

Additional Comments:_____

Recorded by:_____ Date:_____

Figure 6 Example of a suction lysimeter sampling form

5 Summary

The goal of a groundwater monitoring plan for pesticides is to produce water samples that are representative of the aquifer under study at the time of sample collection. Materials and methods must be established prior to implementing a monitoring program

to ensure consistency of sample collection and to permit the comparative analysis of water-quality data. Any deviations from the established monitoring plan must be documented. It is imperative that all personnel participating in the monitoring program are adequately trained and that study protocols and standard procedures are available at all times for review.

Methodologies and materials for developing groundwater and soil-water sampling procedures have been presented. Sources for the collection of groundwater samples have been discussed, in addition to groundwater and soil-water sampling procedures. The discussions included the selection of well materials, the installation of screened and open-hole monitoring wells, and the installation of suction lysimeters. Also discussed were procedures for the purging, sampling, and processing of groundwater and soil-water samples as part of a groundwater monitoring plan. The collection of groundwater sources of springs, seeps, and drain tiles was briefly reviewed.

References

1. US Environmental Protection Agency, Guidance for Prospective Ground-Water Monitoring Studies, Ground-Water Programs, Office of Pesticide Programs, Washington, DC (1998).
2. Ohio Environmental Protection Agency, 'Technical Guidance Manual for Hydrogeologic Investigations and Ground Water Monitoring', Ohio Environmental Protection Agency Division of Drinking and Ground Waters, Columbus, OH (1995).
3. US Environmental Protection Agency, RCRA Ground-Water Monitoring: Draft Technical Guidance, Office of Solid Waste, Washington, DC (1992).
4. L. Aller, T.W. Bennett, G. Hackett, R.J. Petty, J.H. Lehr, H. Sedouis, D.M. Nielson, and J.E. Denne, 'Handbook of Suggested Practices for the Design and Installation of Ground-Water Monitoring Wells', National Water Well Association, Dublin, OH (1989).
5. C.W. Fetter, 'Contaminant Hydrogeology', Macmillan, New York (1995).
6. F.G. Driscoll, 'Groundwater and Wells', Johnson Filtration Systems, St. Paul, MN (1986).
7. US Environmental Protection Agency, Region 4, Environmental Investigations Standard Operating Procedures and Quality Assurance Manual, Sections 1–18, EPA, Athens GA (1996 with 1997 revisions).
8. W.W. Lapham, F.D. Wilde, and M. T. Koterba, Ground-Water Data-Collection Protocols and Procedures for the National Water-Quality Assessment Program: Selection, Installation, and Documentation of Wells and Collection of Related Data, Open-File Report 95-398, US Geological Survey, Reston, VA (1995).
9. D. Hillel, 'Environmental Soil Physics', Academic Press, London (1998).
10. Florida Department of Environmental Protection, 'Standard Operating Procedures for Field Activities', Tallahassee, FL (2002).
11. F.D. Wilde, D.B. Radtke, J. Gibs, and R.T. Iwatsubo, 'Collection of Water Samples', in "National Field Manual for the Collection of Water-Quality Data", US Geological Survey Techniques of Water Resource Investigations, US Geological Survey, Reston, VA, Book 9, Chapter A4, (1999).
12. US Environmental Protection Agency, Region II, 'Ground Water Sampling Procedure – Low Stress (Low Flow) Purging and Sampling', 7 (verified 10/19/01). Also available on the World Wide Web: **http://www.epa.gov/r02earth/smb/lowflow.txt.**
13. F.D. Wilde, D.B. Radtke, J. Gibs, and R.T. Iwatsubo, 'Field Measurements', in "National Field Manual for the Collection of Water-Quality Data", US Geological Survey Techniques of Water Resource Investigations, US Geological Survey, Reston, VA, Book 9, Chapter A6 (1998).
14. F.D. Wilde, D.B. Radtke, J. Gibs, and R.T. Iwatsubo, 'Processing of Water Samples', in "National Field Manual for the Collection of Water-Quality Data", US Geological Survey Techniques

of Water Resource Investigations, US Geological Survey, Reston, VA, Book 9, Chapter A5 (1999).
15. M.T. Koberta, F.D. Wilde, and W.W. Lapham, Ground-water Data – Collection Protocols and Procedures for the National Water-Quality Assessment Program – Collection and Documentation of Wells, and Collection of Related Data, Open-File Report 95-399, US Geological Survey, Reston, VA (1995).
16. F.D. Wilde, D.B. Radtke, J. Gibs, and R.T. Iwatsubo, 'Cleaning of Water Sampling Equipment', in "National Field Manual for the Collection of Water-Quality Data", US Geological Survey Techniques of Water Resource Investigations, US Geological Survey, Reston, VA, Book 9, Chapter A3 (1998).
17. L.G. Wilson, D.W. Dorrance, W.R. Bond, L.G. Everett, and S.J. Cullen, 'In Situ Pore-Liquid Sampling in the Vadose Zone', in "Handbook of Vadose Zone Characterization and Monitoring", ed. L.G. Wilson, L.G. Everett, and S.J. Cullen, CRC Press, Boca Raton, FL, Chapter 26, pp. 477–521 (1995).
18. Soilmoisture Equipment Corp., Operating Instructions for the Pressure-Vacuum Soil Water Sampler, Soilmoisture Equipment Corp., Santa Barbara, CA (1997).
19. Timco Manufacturing Inc., 'TIMCO™ Lysimeter Manual', Timco Manufacturing Inc., Prairie du Sac, WI (1992).

Preparation and instrumental analysis of agrochemical residues in water samples

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1 Introduction

This chapter discusses analytical methodology used for the analysis of pesticides and degradation products in environmental water samples. Surface water from runoff found in ponds, streams, rivers or lakes may contain agrochemical residues due to normal usages or as a result of accidental release. Subsurface water may also be subject to monitoring programs since the agrochemical and its environmental degradation products may leach from the surface into groundwater used as drinking water. A brief overview of the past and current water regulatory issues is also presented. A review of historical methods and state-of-the-art methods employed for water analysis are discussed.

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From a technical standpoint, this article emphasizes recent advances in sample preparation and instrumentation. A brief history of modern sample preparation techniques is covered, together with the impact of modern instrumentation on water sample analysis.

Gas chromatography and liquid chromatography are discussed briefly, but the main focus is centered on the modern aspects of liquid chromatography (LC) coupled with mass spectrometry (MS) and tandem mass spectrometry (MS/MS), and the impact these instruments have had on environmental water analysis. The main focus is on LC/MS and LC/MS/MS due to an increasing need for efficiency in analysis, confirmation and quantitation of polar and thermally labile analytes. A synopsis of the procedures used when employing LC/MS/MS is covered, including the use of stable isotope internal standards and the determination of method detection limits. A brief discussion also outlines the economic benefits of using modern instrumentation. Although such modern instrumentation as LC/MS/MS is expensive, time savings from sample preparation, in addition to improved accuracy, selectivity and sensitivity, far outweigh the initial expense in a brief period of time.

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1.1 Regulatory issues

Regulation of pesticides began in the USA with the passage of the Insecticide Act of 1910. The aim of this Act was to protect people against fraudulent claims and risks to human health. Direct regulation of drinking water came much later with the passage of the Safe Drinking Water Act (SDWA) in 1974. It gave the Environmental Protection Agency (EPA) the legal right to set standards for contaminants in drinking water and the authority to enforce them through fines or other legal action. Internationally, passage of the Drinking Water Directive (80/778/EEC) by the European Union (EU) in 1980 provided the means to improve water quality in the EU. This Directive set limits for pollutants (including pesticides) in drinking water to be achieved by 1985. Each member country was to enact legislation to abide by and to enforce the Directive. In contrast to the EPA guidelines, the EU policy was dependent on the individual member countries to enforce the mandates. Since the EU is a less closely bound political organization than the individual states in the USA, which report to a central government body, enforcement of the mandates often met with political difficulty.

Further legislation in the USA led to an amendment to the SDWA in 1986. It increased the number of potential contaminants that could be regulated. The 1986 amendment augmented the federal role of control by providing for the development of programs to protect groundwater and by monitoring to establish pollution patterns of potential water supply contaminants not under regulation. In 1996 another set of significant amendments to SDWA was implemented. These amendments created a new focus on setting the contaminant regulation priorities based on data about adverse health effects. An estimate of the public health risk was a major focus of the regulations. The States were also given more flexibility to meet their specific needs while arriving at the same level of public safety. A principal goal of the 1996 amendment was to prevent pollution of water sources rather than mitigation of a cleanup later. This was also the goal of the Pollution Prevention Act of 1990, i.e., to prevent pollution rather than to have to deal with massive clean-ups after they occurred.

The EU Drinking Water Directive of 1998 (98/83/EC) was set forth to update the Directive of 1980. The updated Directive took into consideration the most recent technical and scientific information and also provided more information to the public. Another major point was to clearly define compliance at the consumer level. The 1998 Directive was to be written into law by the member states by December 25, 2000 with full compliance in effect by 2003. Acceptable agrochemical residue drinking water limits have been set to $0.1\ \mu g\,L^{-1}$ ($0.5\ \mu g\,L^{-1}$ total) with several specific compounds (aldrin, dieldrin, heptachlor and heptachlor epoxide) set to $0.03\ \mu g\,L^{-1}$. The 1998 Directive is more pragmatic in its approach to compliance. It separates those parameters which are mandatory for human health from those which are significant for esthetic value and therefore nonmandatory in nature. Monitoring at the consumer water tap was also emphasized to improve the practicality of the mandate parameters. This pragmatic approach hopefully will assuage EU members to comply more heartily with the Directive mandates.

In the USA, the passage of the Food Quality Protection Act (FQPA) of 1996 has had a significant impact on the determination of residues in drinking water. FQPA requires that all sources of a pesticide be included in its risk assessment, so the potential exposure from drinking water containing a particular pesticide could be a significant

factor in its marketability. Therefore, FQPA has enhanced the importance of accurate residue methods. It is important that the residue method produce data that is more precise and accurate with a reduced amount of uncertainty. It is very important that the determined residue values not be over or understated because populations generally consume large volumes of drinking water. An inaccurate residue measurement could lead to costly errors involving a compound's risk assessment. Such faulty assessments could lead to costly monitoring programs or inaccurate health related concerns. After the 1986 amendments to the SDWA, the Office of Pesticide Programs (OPP), under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), initiated a program for ground water monitoring for pesticides and their metabolites which may have a potential to leach into the groundwater. The leaching potential is determined by laboratory and preliminary field monitoring studies. The study data help regulators understand the potential for a pesticide or toxicologically significant metabolite to contaminate drinking water, impact groundwater quality and reach ecologically important surface waters. Depending on the expected use profile of a pesticide, an aquatic dissipation study may also be required. This study will require a water method capable of analyzing water in an environment such as a rice paddy. After a pesticide has been registered it is possible that monitoring of both well and surface water may be required.

Monitoring for water quality has also been implemented in Japan. The Japan Water Research Center (JWRC) implemented the Hazardous Chemicals Monitoring and Information Network Project. This project, funded by the Ministry of Health and Welfare (MHW), is a nationwide monitoring effort to track 121 water quality parameters including the measurement of agrochemicals in groundwater. One of the goals is to evaluate the information in the database to try to target possible sources of pollution such as golf courses.

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In summary, environmental legislation by world governments has placed great demands on the scientific community to develop sensitive, cost-effective analytical methods to measure pollutants in all types of water samples. As can be witnessed in the following sections, great efforts have been spent in developing preparatory techniques and analytical instrumentation to satisfy the stringent requirements of these regulations. Water methods have to be able to determine analytes down to low parts per trillion (ppt) (ng L^{-1}) levels and be specific enough to measure individual degradation products of potential pollutants.

1.2 Historical perspectives

One of the early records of analysis of pesticides in water was by Hindin *et al.*[1,2] Paper chromatography was used to analyze chlorinated and organophosphate pesticides. An enzymatic method (cholinesterase inhibition) was used to detect organophosphates and carbamates. Gas–liquid chromatography (GLC) with a thermal conductivity cell detector was used as the confirmatory procedure. The sensitivity was 500 μg with the conductivity detector, but when using a hydrogen flame detector, the sensitivity improved to 1 μg. Several years later, Devine and Zweig[3] reported the analysis of chlorophenoxy ester herbicides in water. The samples were extracted with toluene and determined by GLC using a ^{63}Ni electron capture detector. By the late 1960s, GLC was being put to widespread use for the analysis of pesticides in all types of matrices. For

more than two decades, GLC became the preferred instrumental technology for the measurement of pesticide residues. Refinement of instrumental electronics and the development of specific detectors such as the nitrogen–phosphorus detector (NPD) dramatically increased the performance and acceptance of gas chromatographic systems.

The development of solid-phase extraction (SPE) absorbents such as silica gel, alumina and Florisil tremendously aided in the purification or 'cleanup' of pesticide residues from water.

Law and Goerlitz[4] in 1970 reported the effective removal of co-extractives from water using microcolumns of these three adsorbents for the analysis of chlorinated pesticides. The development of polystyrene resins such as XAD increased the ability to concentrate pesticide residues from water. Large volumes of sample water could be passed through an XAD resin and the pesticide would adsorb on the resin. Elution of the pesticide by an organic solvent such as methanol and subsequent cleanup by the adsorbent materials became the industry standard.

The introduction of the quadrupole mass spectrometer coupled to a gas chromatograph as a commercial instrument in the 1970s proved to be a powerful analytical tool for residue analysis. The fast scanning speeds available on the quadrupole ion filter made it a perfect match for interfacing to a GLC system. This led to a new era in residue analysis providing new realms of selectivity and sensitivity. The subsequent development of the triple quadrupole mass spectrometer by Yost and Enke[5] increased the ability of an analyst to measure pesticide residues in complex matrices with minimal cleanup and improved sensitivity and accuracy. The 1980s brought the development of high-performance liquid chromatography (HPLC) or liquid chromatography (LC) as a powerful tool for residue analysis. The coupling of HPLC to MS by thermospray (TSP) introduced by Blakley *et al*.[6] and later electrospray ionization (ESI) by Whitehouse *et al*.[7] provided the tools to measure chemical pollutants accurately over a much broader range of polarities. The combination of ESI with multi-stage MS has become one of the standard analytical instruments in residue analysis for the twenty-first century.

2 Sample preparation

This section discusses treatment of the water samples in preparation for instrumental analysis after they have been received, archived and stored in the laboratory. Many approaches may be taken in preparation of water samples for final analysis. The techniques employed will depend upon the type of matrix, e.g., groundwater vs surface water (containing organic materials), the instrumental method and the required detection limits.

Many early procedures used for the analysis of water samples utilized organic–aqueous partitions of nonpolar target compounds into the organic fraction, subsequent concentration and analysis. The principles of chromatography introduced by Tswett early in the twentieth century and the use of sorbent materials was first put into practice by Braus *et al*.[8] in 1949. They used an iron cylinder of activated carbon to concentrate organic compounds from raw surface water. Samples were dried and extracted with diethyl ether and the extracted organics were chemically characterized. Another early application of solid sorbent used for analyzing pesticide residues was the use of alumina coated with polyethylene by Hoskins *et al*.[9] Ironically, the procedure held

up the interfering extractives while allowing the pesticide to pass through unretained when using acetonitrile–water mixtures as eluents.

Liquid–liquid extraction was and still is a widely used extraction procedure. Trace amounts of analytes were extracted by Wheatley and Hardman[10] using hexane in 1965. These extracts were then analyzed by thin-layer chromatography (TLC) using silica gel G. Benzene was another popular organic solvent used to extract pesticides. Organochlorine and organophosphorus insecticides were extracted with benzene as reported by Pionke *et al.*[11] The insecticides were then analyzed by GLC using either an electron capture or thermionic detector. Activated silica gel was also used in an early cleanup procedure for samples extracted by liquid–liquid extraction as reported by Kadoum.[12] The unpredictable characteristics of activated carbon led to more widespread use of other more homogeneous sorbent materials such as alumina, silica gel and Florisil. The binding and elution characteristics of these sorbent materials were much more predictable, leading to improved reproducibility of analytical procedures. These sorbent materials were principally used in a cleanup step after the pesticides had been extracted from water by a liquid partition. In the mid-1960s the introduction of the cross-linked polystyrene resin, Amberlite XAD-1, provided a more rugged sorbent material for the direct concentration of pesticides from water. Like activated carbon, water could be passed through a column packed with XAD resin directly. Riley and Taylor[13] were among the early investigators to use this procedure with 1-cm diameter columns packed with 7 cm of XAD-1 resin. Seawater samples were percolated at a rate of 5 mL min^{-1} through the columns. The organic materials were then eluted with 10-mL aliquots of ethanol, dilute nitric acid and dilute potassium hydroxide. The introduction of the ethylene dimethacrylate resins XAD-7 and XAD-8 increased the usage of these polymers. These Amberlite resins were able to retain weak organic acids and bases and also neutral organic compounds, further increasing the polarity range of water-extractable organic compounds and also as more polar pesticides. Some work was done by Gesser *et al.*[14] using porous polyurethane foam to extract polychlorinated biphenyls (PCBs) from water. Adsorption columns of Tenax–Celite were also used by Leoni *et al.*[15] with some success to extract pesticides and PCBs from water.

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These are just a few of the examples of materials used for the extraction of organic compounds from water tested during the 1970s. The application of the Amberlite XAD resins were by far the most numerous, as evidenced by the number of references in an overview published by Junk.[16] Amberlite XAD-4 was used by Musty and Nickless[17] to recover chlorinated insecticides and PCBs from water. Only aldrin and dichlorodiphenyldichloroethylene (DDE) were not extracted quantitatively. Carbamates were extracted from natural waters by Sundaram *et al.*[18] using XAD-2. The water was percolated through a column packed with XAD-2 and eluted with ethyl acetate. Sixteen organophosphorus insecticides were extracted from drinking water by Lebel *et al.*,[19] again using XAD-2 resin and percolating 100–200 L of water through the resin columns. The pesticides were then eluted using acetone–hexane (3 : 17) and analyzed by GLC. Recoveries were greater than 90% except for dimethoate and phosphamidon (37% and 42%, respectively). Although the Amberlite resins gained wide acceptance, because of the presence of artifact compounds such as naphthalene, styrene, hydrocarbons and phthalates, the resins had to be purified. This led to unacceptable, reproducibility of method results between laboratories.[20]

The introduction of HPLC led to the development of silica bonded phases as early as the mid-1970s. Little and Fallick[21] were among the first to recognize that these bonded phases could be used for 'total extraction' of organic compounds from water. The octadecyl bonded phases became the most popular but other alkyl and aryl groups (C_2, C_4, C_8, cyclohexyl, diol, cyanopropyl and phenyl, among others) were also tested. The sorption characteristics, the ability to use polar and nonpolar solvents and improved reproducibility made these column packings instantly popular as SPE media. This popularity extends to the present time as SPE is the most popular current technique for extracting pesticides from water. Early comparisons of SPE with liquid–liquid extraction showed SPE to be as much as 20% better than accepted traditional extraction procedures.[22] The commercial introduction of brand names such as Sep-Pak and Bond Elut increased the popularity of SPE extraction cartridges. The uniformity of the pre-packed columns and the ease of use of the commercially available cartridges greatly expanded their use.

There was also considerable interest directed towards the development of uniform carbonaceous sorbent material during the 1980s. These homogeneous carbonaceous materials exhibited more reproducible results than those of their activated carbon predecessors. The advantage over bonded phase SPE packings is the carbon packed material's ability to retain smaller and more polar compounds. Bacoloni *et al.*[23] reported the extraction of organochlorine pesticides using graphatized carbon black. The pesticide analytes were eluted from the packed carbon columns with hexane–diethyl ether (1 : 1). More recently, Di Corcia *et al.*[24] reported the use of Carbograph 4 to extract atrazine and six of its degradation products from water. Up to 1 L of river water and 4 L of both groundwater and drinking water were extracted. Using LC/MS analysis the analytes could be measured at levels of 40–300 $\mu g\ L^{-1}$ with recoveries for all analytes better than 80%. The same group[25] extended the utilization of Carbograph 4 to extract a number of pesticides and their metabolites from several natural water sources. The extracted samples were then analyzed by LC/ESI-MS.

The introduction of membrane extraction disks in the late-1980s brought about a revolution in the extraction and concentration of pesticides from water. The membranes are a polytetrafluoroethylene (PTFE) matrix in which sorbent materials such as bonded silicas, polymers or ion-exchange materials are attached. The disks are capable of higher flow rates of water sample percolation than extraction cartridges and avoid the channeling problems that occur with cartridges. Hagen *et al.*[26] were among the first to use SPE disks to remove PCBs, pesticides and polychlorinated dibenzofurans from reagent graded water. Advantages were reported to be smaller volumes of solvent used for extraction, reduced time for extraction and, compared with liquid–liquid extraction, the lack of emulsion formation. Brouwer *et al.*[27] reported the use of alkyl bonded silica and ion-exchange material to enrich polar pesticides from surface water. Basic compounds such as carbendazim, chloridazon, simazine and 4-chloroaniline were extracted from acidic solution using one or two extraction disks in series, i.e., operated in the reverse-phase and ion-exchange modes. These compounds were extracted using aqueous acetonitrile mixtures, adjusted to pH 3, as the eluent. The concentrated samples were analyzed by LC. C_{18} Empore disks were also used to extract chlorotriazines, organophosphates, phenylurea and carbamate insecticides from river water.[28] The use of extraction disks allowed for higher flow rates than using extraction cartridges. The pesticides were then detected by using

LC/TSP-MS. The limits of detection were reported to be 2–10 $\mu g\,L^{-1}$. A novel use of extraction disks was reported by Koskinen and Barber[29] to extract atrazine, acetochlor and alachlor from water as a function of time, degree of mixing and disk size. C_8 extraction disks (47-mm) were simply dropped into 500 mL of sample water for 20 h. The adsorbed pesticides were then eluted using ethyl acetate in the sample collection bottle. Recoveries >70% were reported for all the herbicides. This unique procedure eliminated the need for expensive vacuum filtering glassware while decreasing sample handling.

The effects of water temperature and pesticide concentration on pesticide recoveries were tested by Moye *et al.*[30] The pesticides included alachlor, atrazine, bromacil, chlorothalonil, chloropyrifos, diazinon, endosulfan, simazine and trifluralin. Temperatures of 5, 25 , 45 and 65 °C were tested and concentrations of 0.1, 1.0 and 10 $\mu g\,L^{-1}$ were used. Water temperature had a pronounced effect on the recoveries whereas the concentration did not seem to have as great an effect.

Kicuchi and Saito[31] used carbon Empore disks in combination with SDB-XD Empore disks to extract polar (methamidophos, acephate and trichlorfon) and nonpolar pesticides (diazinon, chloroneb and simazine) from water. The water sample (500 mL) was passed through the disk and the disk simultaneously eluted with 30 mL of acetone–ethyl acetate (1 : 1). The samples were concentrated and analyzed by GLC/MS.

SPE extraction disks are the latest in a long line of developments involving SPE from nonuniform carbon packings, through alkyl bonded phase silicas among other polymeric packings exhibiting ion-exchange characteristics to modern uniform carbon packings such as Carbograph 4. Since the early 1990s, developments in the automation of the extraction process have been extensive. On-line extraction using all of the previously mentioned SPE media has been developed. Manufacturers began to produce microprocessor-controlled 'extraction stations' to accommodate large numbers of these cartridges. Vacuum manifold holders became popular as a means to be able to extract large numbers of samples. Other manufacturers such as Zymark, Varian and Merck developed automated SPE extraction stations which were controlled by a personal computer (PC). These stations are capable of holding a series of extraction tubes that can automatically be reproducibly conditioned with the appropriate solvents. The device then siphons the water sample through the equilibrated extraction tube via the use of a syringe pumping mechanism. Generally volumes of sample up to 500 mL are loaded. Once the sample has been loaded, the eluting solvents are automatically added and the samples are collected into a test-tube.

In the last several years, 'on-line' extraction systems have become a popular way to deal with the analysis of large numbers of water samples. Vacuum manifolds and computerized SPE stations were all considered to be 'off-line' systems, i.e., the tubes had to be placed in the system rack and the sample eluate collected in a test-tube or other appropriate vessel. Then, the eluted sample had to be collected and the extract concentrated and eventually transferred to an autosampler vial for instrumental analyses. Robotics systems were designed to aid in these steps of sample preparation, but some manual sample manipulation was still required. Operation and programming of the robotic system could be cumbersome and time consuming when changing methods.

During the 1990s, much attention has been shifted to the development and utilization of on-line systems of extraction and analysis. The evolution of computers and

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computer programs and HPLC valve and column automation systems have greatly aided in these on-line extraction developments. Little and Fallick[32] were the first to propose what has been called 'trace enrichment' for concentrating low levels of non-polar organics from water on the head of an HPLC column. The earliest attempts at on-line extraction were done by simply injecting large volumes of water containing a low level of analyte on to the HPLC column and concentrating the sample on the head of the column. The analyte was chromatographed on the column by increasing the solvent strength of the HPLC mobile phase. The performance of the HPLC column could often be compromised by highly contaminated samples which would degrade the column performance or create high back-pressures. Small guard columns were then employed to 'trap' the analyte contained in the water sample. These small trapping columns protected the principal analytical column and could be replaced periodically at a much lower cost.

A commonly used packing for concentration was PRP-1. This styrene–divinylbenzene copolymer was a popular HPLC column packing used for applications where wide variations in pH may be experienced. The PRP-1 packing was used by Liska *et al.*[33] to trap more than 50 different pesticides from river water. The PRP-1 packing tended to retain the pesticides more tightly than a C_{18} packing and was characteristically more rugged, making an ideal medium for trace concentration. The development of instrumentation such as the Prospeckt cartridge-exchange/solvent selection/valve switching unit by Varian made on-line extraction techniques commercially available. This system could be loaded with a number of different cartridges and the water sample loaded and automatically injected into an HPLC system. Generally, C_{18} alkyl bonded phase cartridges were used with the system. SPE extraction disks were well suited for use with on-line extraction procedures. As early as 1990, Brouwer *et al.*[34] reported the use of octadecyl-modified silica membrane extraction disks to isolate polar pesticides from water. The samples were analyzed by HPLC using an isocratic mobile phase with PLRP-S as the column stationary phase. In a unique, but not widely accepted, technique, vander Hoff *et al.*[35] used automated micro liquid–liquid extraction to extract polar pesticides from water with methyl *tert*-butyl ether. This system was used to extract 1 mL of water, and then the extract, was subsequently concentrated and analyzed by GLC using an NPD or flame photometric detector.

Chiron and Barcelo[36] used C_{18} Empore disks to extract pesticides on-line from drinking water. They extracted assorted carbamate insecticides and the herbicides chlortoluron, isoproturon and metolachlor from water followed by fast scanning ultraviolet/visible (UV/VIS) and post-column fluorescence detection. Chiron *et al.*[37] also reported the use of LC/TSP-MS to analyze environmental waters using the same on-line system. Lacorte and Barcelo[38] reported the used of LC/TSP-MS to analyze organophosphorus pesticides and their transformation products in river waters using on-line SPE. They used C_{18} precolumns with an automated on-line precolumn exchange system (OSP-2) for trace enrichment followed by selected ion monitoring (SIM) of thermospray positive or negative ions. A 100-mL volume of water was extracted, resulting in a limit of detection (LOD) of 0.01–0.1 $\mu g\,L^{-1}$.

Activated carbon disks have also been used for the extraction of polar pesticides from environmental water samples. Slobodnik *et al.*[39] used an Empore activated carbon disk (EACD) and Envi-Carb graphitized carbon black (GCB) and CPP-50 graphitized carbon for the trace enrichment of polar pesticides from water.

Comparisons were made to off-line versus on-line extraction as well as testing breakthrough volumes and recoveries. The EACD cartridges performed better than the Envi-Carb cartridges with respect to breakthrough during the off-line comparisons. The Envi-Carb cartridges could also not be used during the on-line applications because of poor pressure resistance. However, the CPP-50 cartridges also provided less peak broadening than the EACD cartridges during the on-line experiments. Overall it appears that the EACD disks may perform best for 'off-line' applications while the CPP-50 disks may be the choice for 'on-line' methods.

Polymeric precolumns of styrene–divinylbenzene were used by Aguilar *et al.*[40] to monitor pesticides in river water. Water samples (50 mL) were trace enriched on-line followed by analysis using LC combined with diode-array detection. LC atmospheric pressure chemical ionization (APCI) MS was used for confirmatory purposes. It was found that after the pesticides had been extracted from the water sample, they could be stored on the precartridges for up to 3 months without any detectable degradation. This work illustrates an advantage of SPE for water samples. Many pesticides which may not be stable when stored in water, even at low temperature, may be extracted and/or enriched on SPE media and stored under freezer conditions with no detectable degradation. This provides an excellent way to store samples for later analysis.

A novel, highly selective trace enrichment on-line cleanup procedure for triazines has been reported by Koeber *et al.*[41] The procedure is based on the use of a molecularly imprinted polymer (MIP) as an SPE material combined with restricted access material (RAM). The method employs a combination of RAM and MIP material, allowing the sample preparation to be done in an on-line mode. The RAM column combines size exclusion and adsorption chromatography and reduces matrix molecules with a 15 000 Da cutoff. The MIP column is designed to retain triazine analytes selectively whereas the matrix residue is not retained. Thus, automated RAM/MIP is capable of excluding all matrix and nontarget compounds. The purified and enriched samples are then analyzed by LC/MS. The complete multi-dimensional SPE extraction, separation and detection takes less than 15 min to analyze tertbuthylazine, atrazine, propazine, simazine, ametryn, prometryn, irgarol and the metabolites deethylatrazine and deisopropylatrazine without any matrix interference from such common contaminants as humic acids. The method was tested on a number of river water samples, and the polymer stability was tested on more than 300 consecutive injections.

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In summary, the development of materials for the extraction of pesticides from water samples has progressed from simple liquid–liquid extraction for the principal active compound to sophisticated SPE media capable of exclusively trapping the target pesticide and metabolites selectively. The development of alkyl bonded phase silica cartridges and extraction disks combined with on-line extraction techniques is currently the principal means used for the extraction and trace enrichment of pesticides and metabolites from water.

3 Instrumentation

3.1 Historical perspective

As dramatic as the developments in the area of SPE media have been over the last 40 years, the development of instrument technology has even made greater

advancements. The need to measure pesticide residues became significant after the passage of FIFRA and the requirements to determine residues in food products for tolerances were established in the 1950s. GLC or gas chromatography (GC), initially developed as an viable technique by Bobleter,[42] was the first significant advancement in instrumentation to gain widespread use as an analytical tool to measure pesticide residues in crops, water and soil. Through developments in electronics and detectors in the 1950s and early 1960s, GLC became the flagship instrument for residue analysis by the mid-1960s. Early chromatographic separations were performed on glass or metal columns packed with an inert solid support coated with a thin coat of silicone-containing liquid. The mobile phase was usually a carrier gas of either helium or nitrogen. As the gas passed through the column, molecules of analyte would partition at different rates between the flowing gas and the thin liquid film on the solid support, thus providing selective retention of the analytes of interest. Advancements in column design in the 1970s saw the introduction of long, narrow-bore capillary columns made of fused silica. The liquid phase was layered on to the fused silica, eliminating the need for a particulate solid support. These columns provided greatly improved resolution with the ability to have long analysis times to separate complex matrices.

Detector technology played a significant role in the development of analytical instrumentation. The flame ionization detector (FID)[43] was one of the first detectors developed. Although an adequately sensitive detector for its time, the FID suffered from the lack of specificity for target compounds. The hydrogen flame tended to oxidize all of the compounds from the column effluent, acting principally as a proportional mass detector, resulting in responses that were proportional to the mass of the compound being measured.

Later the development of detectors specific to certain atoms of a compound greatly enhanced GLC as a tool for trace residue analysis. The electron capture detector (ECD) was developed to have enhanced selectivity for electronegative compounds, specifically those containing halogen atoms. The flame photometric detector (FPD) was developed to have preferential response to those pesticides containing phosphorus and sulfur. Probably the most widely used detector in the pesticide industry is the NPD. Depending on the flow rates of the gases (nitrogen or helium as the mobile phase and hydrogen) the detector can be optimized to detect preferably nitrogen- or phosphorus-containing compounds. Today, along with the ECD, the NPD is still one of the most commonly used detectors.

In the early 1970s, the introduction of the quadrupole mass spectrometer changed the landscape of residue analysis in the coming decades dramatically. The combination of GLC with the mass spectrometer as a detector proved to become the major tool for residue analysis for the next 20 years.

The quadrupole mass detector was a good match for GLC because the mass range could be scanned fast, so sharp GLC peaks produced by the newly developed fused-silica capillary columns could be sampled frequently enough to produce statistically sound data. The analyzer chamber was compact so the gas from the GLC column entering the mass spectrometer vacuum could be pumped away readily. The development of the computer data system for mass spectrometer data also aided in the ability to handle the large volume of data points collected. Finnigan Corporation of San Jose, CA, was among the first manufacturers to introduce a commercially available instrument with the Model 1015. Later, Hewlett-Packard (now Agilent) of Palo Alto, CA, introduced a bench-top model designed specifically as a GLC detector. The

Model HP 5890 was specifically designed for residue analysis and gained widespread acceptance as a standard instrument for GC/MS residue applications.

The development of HPLC during the 1970s proved to be a major breakthrough in technology for the field of analytical residue analysis. For the first time, compounds which could not be measured directly because they were either too polar, labile or difficult to derivatize were able to be quantitated. Although reverse- and normal-phase column packings were tested, the development of stable C_8 and C_{18} reverse-phase alkyl bonded phase column packings, which could withstand the rigors of high pressure, was a major contributor to the success of HPLC. The development of small particle size materials also increased the number of theoretical plates of a column, allowing for much better peak resolution during a chromatographic separation. Significant pioneering work was done by Kirkland[44] at DuPont, resulting in the development of stable bonded silica stationary phases. Most of the original HPLC hardware was developed by the Waters Corporation of Milford, MA. The pumping systems designed by Waters to deliver high pressures and stable flow rates were an important key to the widespread acceptance of HPLC. The original detectors were simple flow-through ultraviolet (UV) detectors. As detector technology development advanced, variable-wavelength UV detectors were introduced. The photodiode-array detector was developed which could scan several wavelengths at the same time allowing only the detection of compounds with the unique pattern. These developments, along with the introduction of a variety of detectors among which were the fluorescence and electrochemical detectors, provided the analyst with an array of detectors to handle difficult analytical challenges. As with GLC, the desire was great to have an interface to join HPLC with MS. The early 1980s and 1990s saw the introduction of several interfaces designed to handle eluents from HPLC, such as the moving belt interface and later the development and commercialization of TSP by Marvin Vestal. The 1990s brought the development of the atmospheric pressure ionization (API) sources, which allowed HPLC/MS to assume a major role in quantitative and qualitative analysis of small molecules including agrochemicals.

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3.2 Current technology: Mass spectrometry

Although over the past 50 years many types of instrumentation and detectors have been used for residue analysis, the evolution to the increasingly widespread use of MS as the principal and most desirable detection system for chromatographic analyses cannot be dismissed. The discovery of chloroform in drinking water by Rook[44] in the 1970s launched MS as a significant tool for water analysis. The 1976 publication by the American Cancer Institute linking cancer formation to chloroform created a major public health issue and it was clear that MS would play a major role in the identification and quantitation of pollutants for years to come. A publication by Keith[45] in 1976 stated that in 1970 only about 100 organic compounds had been identified in water, but by 1976, several years after MS and GLC/MS had been introduced, nearly 1500 organic compounds had been detected. GLC/MS has become a permanent technology for agrochemical trace analysis in water, especially for monitoring long-established volatile pesticides. Many current regulatory methods are based upon GLC/MS. GLC/MS continues to provide reliable residue data important for numerous

monitoring and regulatory projects. The development of GLC/MS made it clear that the capability and contributions of MS would be a permanent fixture in the world of residue analysis.

The introduction of HPLC in the early 1970s presented many new possibilities for the measurement of water pollutant residues covering a wide range of polarity and not just nonpolar organosoluble compounds possessing the volatility required for GLC or GLC/MS. Unfortunately, the detectors available for HPLC, in general, lacked the sensitivity or specificity that was really required for trace level residue analysis. The UV detectors were and still remain the most popular detectors. The UV detector is the simplest and earliest of the detectors used for HPLC, but for trace level analyses it lacks sensitivity and selectivity. The UV spectrum of most compounds is not sharp enough to produce adequate resolution for good specificity. Also, the UV absorbance for some compounds may be low, resulting in reduced sensitivity. The development of variable-wavelength and photodiode-array detectors enhanced the ability of UV detectors to determine specific compounds, but the general lack of sensitivity of UV detectors, which is as a function of the analyte absorptivity, limited their use in many trace residue applications. More specific and sensitive detectors such as fluorescence and electrochemical detectors were developed to address some of these problems. However, once again they lacked universal use because of specific chemical characteristics of the target compound which were required for detection.

Considering the growing success of GLC/MS, a liquid interface to a mass spectrometer for HPLC was highly sought-after technology. One of the earliest interfaces was developed by Finnigan Corporation with the introduction of the moving belt interface. The use of this interface for measuring organic pollutants in surface water was reported by Schauenburg *et al.*[46] The HPLC column effluent was deposited on a metal alloy belt, the solvent dried by heat and the sample then introduced into the mass spectrometer. This technique met with limited success because of high chemical noise and lack of sensitivity. It was not until the development of TSP by Vestal, as mentioned earlier, that HPLC interfaces became productive and practical.

At Bayer CropScience an HPLC/MS method using TSP was developed for the measurement of a dihydroxy metabolite of anilazine present in soil and aqueous samples in 1990. Numerous attempts had been made to develop a residue method by derivatizing the cyanuric acid moiety with methyl iodide to generate methyl ethers. The derivative would then be measured by GLC with an NPD. The problem was finding conditions that were favorable for the formation of both ethers. Also, the resulting derivative was somewhat labile and irreproducible, making reliable quantitation impossible. An HPLC/TSP-MS/MS method using a triple quadrupole mass spectrometer was developed by Alta Analytical Laboratories, Sacramento, CA, to analyze the samples. The development and validation time for the method consisted of only about 2 weeks of effort. It was clear after this experience that the utilization of HPLC/MS and especially HPLC/MS/MS would play a significant role in the future of residue analysis. Others, such as Volmer,[47] used HPLC/MS with TSP ionization to measure pesticides and their degradation products in aqueous environmental samples down to the parts per trillion (ppt) level. Chiron *et al.*, as mentioned earlier, used on-line SPE to measure 34 pesticides and transformation products. SPE using a PRP-1 precolumn or Empore extraction disks

containing C_{18} were used to extract the compounds, which were ultimately analyzed using TSP via SIM.

Although TSP was perhaps the most successful of the early HPLC interfaces to MS, it was very compound dependent, i.e., displaying exceptional sensitivity for some compounds while producing a limited response for others. Ammonium acetate buffer was used in the LC mobile phase and heat was applied via the TSP vaporizer to aid ionization. The size of the vaporizer exit hole and control of the heating of the vaporizer were critical elements to the success of the TSP interface. One of the most aggravating problems with TSP was the occasional irreversible clogging of the vaporizer outlet orifice. Another issue with TSP stemmed from the heat that was required by the vaporizer. Some thermally labile compounds would degrade upon exposure to the vaporizer, thus limiting its use for the measurement of polar compounds. Finally, ion stability of most of the commercially available TSP interfaces was not adequate for use of the interface for quantitative analysis over extended periods of time. This instability generally was caused by the general lack of control over the ionization process and the deficiency of good designs for vaporizer heaters.

Several other interface designs were introduced over this period, including continuous flow fast atom bombardment (CFFAB)[48] and the particle beam interface (PBI),[49] but it was not until the introduction of the API source that LC/MS applications really came to the forefront for quantitative analysis. Early work by Muck and Henion[50] proved the utility of an atmospheric pressure interface using a tandem quadrupole mass spectrometer.

The early API interfaces created ionization of the HPLC effluent via the use of a corona discharge. A needle was used to ionize the nebulized gas discharged from a heated vaporizer, thus creating a plasma where ionization could occur. Because of its similarities to classical chemical ionization MS, the technique became known as atmospheric pressure chemical ionization (APCI). This technique proved to be a significant improvement over TSP, providing greater sensitivity and stability. It still suffered from the presence of a great amount of heat (the vaporizer is generally operated at several hundred degrees) being applied to the HPLC effluent, which caused thermal breakdown of polar or thermally labile compounds. Theoretically, the process is similar to chemical ionization with the solvent molecules providing the protons for the analyte molecule. The development of electrospray ionization (ESI) provided an ionization technique which solved many of the previous HPLC interface problems. The sensitivity was greatly improved (by at least a factor of 10) over TSP. Since no heat was added to the vaporization process, thermally labile compounds were able to be ionized without decomposition and the ionization process was much more universal than with TSP. An added advantage of ESI is that the signal derived from the target compound is dependent on the concentration of the analyte in the HPLC effluent rather than its total mass. This characteristic allows the technique to be adapted to low flow rates and also allows the HPLC effluent to be split so that only a fraction of the sample injected on to the HPLC column actually enters the API interface. Additionally, only a fraction of the spray (approximately 0.1%) entering the ESI interface is sampled by the inlet to the mass spectrometer. Therefore, the actual analyzer portion of the mass spectrometer is not easily contaminated and years of operation can be performed before cleaning of the analyzer is required.

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Combining API interface technology with MS/MS provided a powerful tool for rapid and sensitive determinations of pesticide residues in water. MS/MS was developed in the early 1980s by Yost and Enke.[5] The analyzer consists of a set of three quadrupole mass analyzers set up in a linear orientation in the mass spectrometer analyzer. The center quadrupole is used as the collision cell and in MS/MS analysis the third quadrupole is used as the product ion analyzer. Normally MS/MS scans are performed by setting the voltage on the first quadrupole to allow the passage of a single precursor ion (usually either the positive or negative pseudomolecular ion of the target compound) into the second quadrupole or collision cell. The collision cell contains argon gas to a pressure of approximately 2.5 mTorr (1 Torr = 133.3 Pa) with an offset voltage usually in the range ±16–30 V to induce degradation and ionization of the precursor ion depending on the mode of operation. The normal vacuum in the analyzer is in the range 10^{-6} Torr. The high pressure and energy (via an offset voltage) introduced in the collision cell break the pseudomolecular ion into various characteristic product ions, which are selectively scanned for in the third quadrupole followed by detection and amplification ultimately via an electron multiplier.

3.3 Selected reaction monitoring (SRM)/confirmation

The instrument scan mode called selected reaction monitoring (SRM) is generally used for quantitative applications. SRM is similar to selected ion monitoring (SIM) in single quadrupole MS. The difference is that a product ion from the decomposition reaction in the collision cell is measured instead of a single ion formed in the instrument's source. The advantage is that the product ion formed when performing SRM scans is much more selective than a single ion monitored in SIM scans, because particular precursor ions only produce product ions characteristic of their structure. The signal-to-noise ratio (S/N) is also generally improved by at least a factor of 10. This S/N improvement stems from the selectivity gained by measuring only product ions from a selected precursor compound. The S/N plays an important role in the sensitivity gains witnessed in SRM. Usually 1–4 product ions are monitored from a specific precursor molecular ion, depending on the number of product ions formed from the precursor ion. Sensitivity is also improved, since the instrument may be set up to monitor a small number of scans for longer times. Since scan time is limited by the desired chromatographic peak width, the fewer the number of product ions measured, the greater the increase in overall sensitivity. For applications where low residue levels must be determined, only one transition or product ion is measured. Confidence in the identity of a measured peak depends on the extent to which the transition is characteristic of the analyte. When a high level of confidence in the identity confirmation of an analyte is required, it is usually necessary to detect at least two, and preferably three, product ions. The ratios between the product ion peak areas can be compared with an authentic standard to determine the presence of the compound. In many situations in MS/MS, three product ions of adequate abundance are not generated. In these cases, if two product ions are present, the ratio between the two ions and the LC retention time can be considered as adequate confirmatory criteria. It must be remembered that the technique of MS/MS is inherently more specific than MS alone, because initially a specific ion is chosen in Q1; hence any signal produced

by the instrument is by definition from a 'selected ion'. Essentially MS/MS begins in specificity where SIM leaves off. This should also be considered when identifying confirmation criteria.

Cairns[51] examined the practical aspects of what should be considered sufficient information for confirmation by MS/MS when using 'soft' ionization interfaces such as API. Considering the added specificity of MS/MS, Cairns suggested that the presence of the molecular ion and one structurally related fragment ion along with a retention time match (±2%) to a standard provide a sound basis for confirmation. This contrasts with the traditional view of having to compare three structurally related ions in traditional electron impact (EI) spectra.[52] In addition, when using deuterated analogs as an internal standard (IS), a match of ±25% of the ion ratio of the IS compared with the native compound should be considered adequate for confirmation.

4 Matrix effects, calibration and quantitation

Even though API source design for HPLC proved to be a dramatic improvement over all previous engineering attempts to interface HPLC with MS, some problematic issues still remain. Highly electronegative molecular species present in the LC effluent can suppress the analyte signals dramatically when compared with a standard in pure solvent by competing for the available charge present in the aerosol spray. This effect is seen dramatically in biological samples such as plant and animal tissue or soil extracts. Water samples are usually considered to be free from many of the matrix components, which could cause this effect, but this certainly is not always the case. Surface water samples can contain algal growth or sediment which can make a water sample behave almost like a plant extract. Dissolved minerals and salts from surface runoff can also cause suppression effects. Even groundwater samples which appear to be pristine can contain dissolved components which can cause a reduced signal. However, suppression effects in groundwater generally can be minimized by simple sample preparation or dilution. Occasionally, it is necessary to facilitate instrumental analysis for water samples because of matrix suppression or general lack of sensitivity. On these occasions, it is necessary to provide sample preparation procedures to remove interferences to minimize matrix effects or simply to concentrate the samples. SPE is the most commonly chosen method for pre-analysis sample preparation. Varying volumes of water can be percolated through SPE cartridges and then eluted with an appropriate organic solvent. Previous discussions on sample preparation have covered the various available materials and techniques. One note of caution, when water samples are extracted using SPE, is that often eluting solvents containing acid or base are often needed to elute the strongly absorbed polar components. Residual traces of the acid or base in the final sample can cause significant signal suppression. Often salts or other polar organic matrix components may be eluted with the target analyte and remain in the final sample solution. If this occurs, the benefits of sample preparation could be compromised.

At Bayer CropScience, the use of a stable isotope IS has become common practice to eliminate the effects of matrix suppression on instrument signals. The stable isotopes are synthesized by deuterium exchange reactions on authentic native standards or the

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replacement of carbon or nitrogen with stable label atoms of ^{13}C or ^{15}N. Generally a mass increase of at least 3 amu is desirable to prevent any contamination or cross-talk with native compound in the mass spectrometer. The use of a stable isotope IS not only compensates for the suppression effects seen in the API interfaces, but also allows for fewer injections to be made during a residue analysis study. The reduction in instrument time needed can provide significant cost savings. The IS is generally added in a constant amount, usually after the water sample is extracted and before it is concentrated to the final volume. Occasionally, when compound stability or extraction recovery becomes problematic, the IS may be added to the sample before extraction. It must be emphasized that if this practice is followed, the amount of analyte present in the final dilution needs to produce an adequately intense signal (with acceptable S/N) to measure reliably the amounts necessary to meet the detection limit requirements.

4.1 Quantitation

Quantitation of agrochemical residues measured by MS/MS is largely accomplished by comparing unknown values with a calibration curve covering the range of expected residues. The calibration curve can be generated in the traditional way using external standard calibration plotting peak areas versus concentration. The peak area of the unknown sample is compared with the calibration curve and the corresponding concentration is determined based on the equation of the line. This procedure can quickly become problematic when sample matrix suppression affects the analyte response. Extensive time-consuming sample cleanup may have to be performed to eliminate the matrix effects in order to achieve accurate measurements, thereby reducing some of the benefits of the MS/MS analysis. As discussed before, the use of a stable isotope IS compensates for the suppression effects. Quantitation with internal standards is accomplished by scanning for the appropriate IS product ions concurrently with native ions. A calibration curve is generated, plotting the concentration versus the ratio between the IS and the native compound. The IS is present in a constant amount [5–10 times the limit of quantitation (LOQ)] while the native standards are prepared at concentrations from the LOQ through the expected concentration range to be measured. After the calibration curve has been prepared, the unknown samples are measured and the ratio of native compound to IS is determined for the target analyte. The concentration is then determined from the calibration curve. Since the ratio of the native compound to the IS is not dependent on the absolute signal and the IS is a stable isotopic congener to the native compound, any suppression of the signal does not affect the final determination of the amount of target analyte.

4.2 Detection limits

The determination of the LOD and LOQ is an important aspect of any method used for water analysis. A review article by Keith[45] outlines many of the issues concerning the determination of method detection limits. In general, the determination of the

LOD and LOQ are best summarized by the American Chemical Society definition. The LOD is defined as 3σ [σ = one standard deviation (SD)] from the mean signal of a well documented signal in a control sample while the LOQ is defined as 10σ of the same value. A drawback to this determination is that the blank must have a signal which can be measured. In MS/MS analyses often there is no measurable signal in the blank or control matrix. The EPA method of estimating the method detection limit (MDL) is gaining wider acceptance. This procedure is outlined in 40 CFR Part 136, Appendix B.[53] Briefly, seven replicates of a control matrix sample are fortified with the target analyte within a factor of five times the MDL (the MDL is similar to the LOD). The residue amounts are determined and the standard deviation calculated. The MDL or LOD is calculated using the equation $\text{MDL} = t_{(N-1,1-\alpha=0.99)} \times \text{SD}$, where t = Student's t-value for a one-tailed test at the 99% confidence level with $N - 1$ degrees of freedom ($t = 3.143$ if $N = 7$), N = number of replicate analyses and SD = the standard deviation of the replicate analyses. The LOQ , however, is defined as three times the LOD or the lowest level a fortified recovery is performed according to regulatory requirements. This level for water methods is usually 0.1 $\mu g\,L^{-1}$.

5 Applications of LC/API-MS and LC/API-MS/MS in water sample analyses

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The use of HPLC/API-MS techniques has become much more widespread in the last few years. Concerns with surface and groundwater contamination and studies mandated by regulatory agencies to protect these precious resources have led to the widespread proliferation of HPLC/MS, because of the selectivity and sensitivity provided by this technology. Hu *et al.*[54] reported the use of HPLC/APCI-MS to measure thermally labile and polar pesticides in untreated and treated water used for a public water supply. The 31 pesticides measured were not amenable to GC/MS analysis because of problematic stability. The pesticides were extracted from water using a special cartridge packed with polystyrene–divinylbenzene in the upper part and carbon molecular sieves in the bottom. Wickremesinhe *et al.*[55] reported the measurement of oxamyl and its oxime metabolite in soil pore water from the saturated and unsaturated zones and also surface irrigation water associated with ground water studies, API in combination with HPLC/MS/MS. The LOD was 0.1 $\mu g\,L^{-1}$ and the LOQ was 1 $\mu g\,L^{-1}$. MS/MS was performed in the SRM mode and provided both confirmatory and quantitative data.

Ingelse *et al.*[56] reported the measurement of six polar organophosphorus pesticides by HPLC/API-MS with direct aqueous injection (DAI). The six compounds, acephate, methamidophos, monocrotophos, omethoate, oxydemeton-methyl and vamidothion, could not be extracted from water using commonly available SPE cartridges. GC analysis was also problematic because the analytes were polar and thermolabile in nature. A 1-mL amount of sample was directly injected on to an RP_{18} HPLC column with polar endcapping under 100% aqueous conditions. This allowed the target

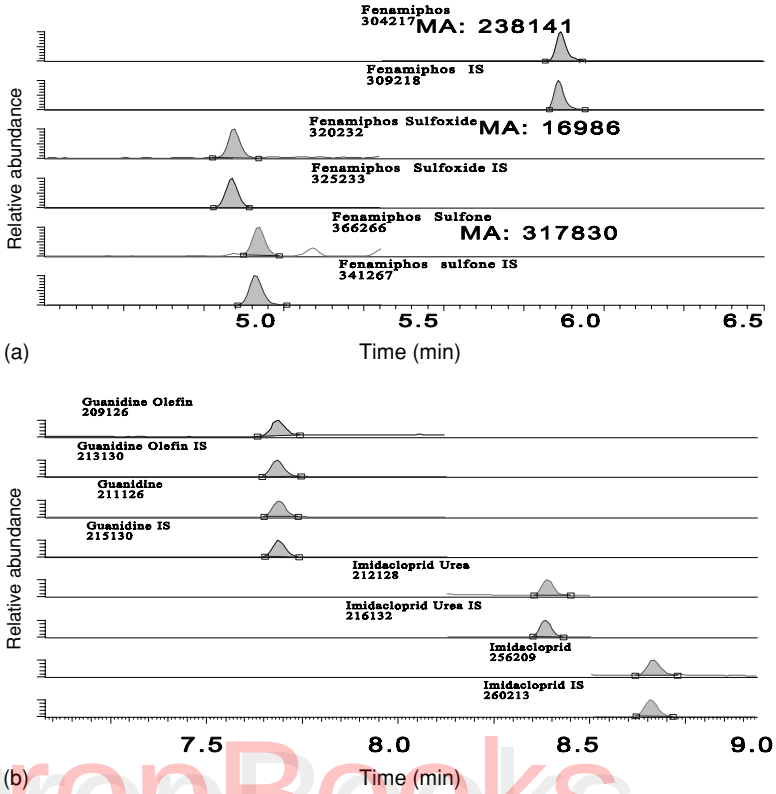
pesticides to be concentrated on the front of the column and later eluted into the mass spectrometer.

The initiation of groundwater studies at Bayer CropScience and the subsequent analytical demands prompted the implementation of HPLC/API-MS/MS for the analysis of the resulting water samples. Requiring the measurement of all metabolites from the environmental studies presented the problem of analyzing a number of compounds with widely varying polarities. The sensitivity required for this analysis was also an issue. The determination of all metabolites down to a residue level of 0.1 $\mu g\,L^{-1}$ presented special problems for conventional analysis, especially since the volume of water sample is sometimes limited. The number of samples generated from a single groundwater site also required an expedient analysis to make the studies cost effective, since it is not uncommon to generate 1200–1500 samples per year. Most studies also require the determination of at least 3–4 metabolites per sample. A groundwater study can easily generate 4000–6000 individual residue determinations per year, so it is easy to see how labor intensive a groundwater study could become if each analyte required a separate analysis method.

Two examples will be presented as an example of groundwater analyses at Bayer CropScience. Both analyses were carried out using DAI with the aid of a stable isotope IS. Attempts at conventional SEP extractions presented special problems because of adsorption of metabolites on the SEP media or oxidative instability during extraction. A brief description of the procedures is outlined below.

Fenamiphos (Nemacur) and metabolites were determined directly by HPLC/API-MS/MS in the electrospray mode. A 1-mL aliquot of the water sample was directly added to a 1.8-mL glass autosampler vial and 200 μL of IS solution, containing each compound as the d_4 stable isotope, was added to each vial. The ISs were present in the sample at a residue level equivalent of 2 $\mu g\,L^{-1}$. A 200-μL volume of the water sample was injected and chromatographed on a 50 × 3.2-mm i.d., 5-μm Prodigy 5 ODS column (Phenomenex, Torrance, CA) using a gradient of water and methanol with the water containing 5 mM ammonium acetate. HPLC/API-MS/MS in the positive ESI mode was performed using one SRM transition from each compound. The residue was determined by measuring the ratios of the native compound to the IS and comparing them with a calibration curve as described earlier. Figure 1(a) shows an example chromatogram of this analysis.

Imidacloprid (Admire, Premise, etc.) is a widely used insecticide for various pests. The parent compound and three metabolites were measured for a prospective groundwater monitoring study. Hydrolysis of the parent compound produced two guanidine metabolites, one containing an additional double bond. These metabolites were difficult to concentrate by SPE without some degree of irreversible adsorption on the SPE material. Chromatographic separation of these compounds was also a challenge. A DAI method was developed similar to the fenamiphos procedure described above. Chromatography was performed on a 150 × 4.6-mm i.d., 5-μm LUNA C_8 column. The mobile phase consisted of water and acetonitrile, both containing 0.1% formic acid. A 15-min gradient was used. Samples were prepared in the same manner as described above for fenamiphos, including the addition of a stable isotope IS for each compound. A 200-μL volume was injected on to the LC column. MS was performed in the SRM mode, monitoring one product ion for each compound, a total of eight including ISs. An example of a chromatogram from an imidacloprid water sample is



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Figure 1 SRM chromatograms of (a) fenamiphos and metabolites and (b) imidacloprid and metabolites. IS refers to the stable labeled isotopes. The values below the names refer to the mass transitions, i.e., M + 1 → product ion for the metabolite

presented in Figure 1(b). The MDL or LOD was determined as described previously using the EPA method employing Student's *t*-test. The LOD for all compounds fell within the range 0.01–0.04 $\mu g\,L^{-1}$. Recoveries for all compounds were successfully performed at the 0.1 $\mu g\,L^{-1}$ level.

6 Conclusion

The evolution of procedures, instrumentation and methods for water analysis have made much progress during the past 50–60 years. Detection techniques which required micrograms of material to detect 60 years ago have been reduced to less than picogram levels with present-day instrumentation. Regulatory demands for analytical specificity and lower detection limits and the improvements in electronics and computer technology have resulted in the design and development of some of the most sophisticated instrumentation of any field of modern endeavor. Combined with the advances in SPE technology, accurate information on water contamination is easier and less expensive to produce than ever before. Although the most sophisticated LC/MS/MS instrumentation can cost several hundred thousand dollars per system, the sensitivity,

Table 1 Comparison of analytical instruments[a]

Instrument type	Specificity/sensitivity	Sample preparation time	Data quality	Cost effectiveness
GC	⋆⋆	⋆	⋆	⋆⋆
GC/MS	⋆⋆	⋆⋆	⋆⋆	⋆⋆
LC	⋆	⋆⋆	⋆⋆	⋆⋆⋆
LC/MS	⋆⋆	⋆⋆⋆	⋆⋆	⋆⋆
LC/MS/MS	⋆⋆⋆⋆	⋆⋆⋆⋆	⋆⋆⋆⋆	⋆⋆⋆

[a]⋆, Least desirable; ⋆⋆⋆⋆, most desirable.

number and complexity of samples and the speed and reliability with which these instruments operate can produce residue information more economically than ever before. Table 1 presents a comparison of the instrumentation used in most water analysis methods, both past and present. GC, GC/MS, LC/UV, LC/MS and LC/MS/MS are compared by categories including specificity/sensitivity, sample preparation time, data quality and cost effectiveness, showing the value of LC/MS/MS for trace analysis. The number of stars in each category represents the desirability of characteristics for that technique in an actual sample matrix. For example, GLC receives a single star in the catagory 'Sample preparation time,' because usually a water sample will have to be extracted, cleaned up and perhaps derivatized before GLC analysis. In contrast, LC/MS/MS receives four stars because often a water sample may be directly analyzed, precluding many of the steps required for GLC.

In most categories where analyses of multiple analytes at low detection limits are required, LC/MS/MS is the technique of choice. However, depending on the type of water analysis being performed, one of the other instrument choices may be more feasible. If, for example, only the parent is to be analyzed and it shows high absorption, HPLC/UV may be a more economical alternative. If less selectivity is required, perhaps GC/MS or HPLC/MS could be a better choice. However, if there is a need for a large volume of samples with multiple metabolites with varying polarities requiring high sensitivity and specificity, generally HPLC/MS/MS is the technique of choice. MS/MS often will preclude the need to develop multiple methods by nonhyphenated techniques. Interferences in sample chromatograms produced by GC or HPLC can lead to the need for multiple purification processes, which require time and money. Metabolites of widely varying polarity could require derivatizations or separate procedures in traditional methods, leading to a single sample requiring three or four separate methods. Development problems of this nature can be eliminated by the use of MS/MS for the analysis.

The nature of water residue analysis has changed dramatically over the past 60 years. Advances in SPE media have made the isolation of 'hard to extract' residues more attainable. The use of specific and sensitive instrumentation as in HPLC/MS/MS has even precluded the need for extraction in many cases, since the samples can be directly analyzed as they are received from the field. The future holds even more improvements in sensitivity with rugged new API interfaces and high-resolution mass spectrometers that will dramatically increase the specificity of detection and ease of analysis.

References

1. E. Hindin and G.H. Dunstan, in 'Proceedings of the British Insecticide and Fungicide Conference,' Brighton, p. 401 (1962).
2. E. Hindin, M.J. Hatten, D.S. May, Jr, R.T. Skrinde, and G.H. Dunston, *J. Am. Water Works Assoc.,* **54**, 88 (1962).
3. J.M. Devine and G. Zweig, *J. Assoc. Off. Anal. Chem.,* **52**, 187 (1969).
4. L.M. Law and D.F. Goerlitz, *J. Assoc. Off. Anal. Chem.,* **53**, 1276 (1970).
5. R.A. Yost and C.G. Enke, *US NTIS, AD Rep.* (AD-A048011) (1977).
6. C.R. Blakley, J.J. Carmody, and M.L. Vestal, *J. Am. Chem. Soc.,* **102**, 5931 (1980).
7. C.M. Whitehouse, R.N. Dreyer, M. Yamashita, and J.B. Fenn, *Anal. Chem.,* **57**, 675 (1985).
8. H. Braus, F.M. Middleton, and G. Walton, *Anal. Chem.,* **23**, 1160 (1951).
9. W.M. Hoskins, W.R. Erwin, R. Miskus, W.W. Thornburg, and L.N. Werum, *J. Agric. Food Chem.,* **6**, 914 (1958).
10. G.A. Wheatley and J.A. Hardman, *Nature (London),* **207**, 486 (1965).
11. H.B. Pionke, J.G. Konrad, G. Chesters, and D.E. Armstrong, *Analyst,* **93**, 363 (1968).
12. A.M. Kadoum, *Bull. Environ. Contam. Toxicol.,* **2**, 264 (1967).
13. J.P. Riley and D. Taylor, *Anal. Chim. Acta,* **46**, 307 (1969).
14. H.D. Gesser, A. Chow, F.C. Davis, J.F. Uthe, and J. Reinke, *Anal. Lett.,* **4**, 883 (1971).
15. V. Leoni, G. Puccetti, R.J. Colombo, and A.M. D'Ovidio, *J. Chromatogr.,* **125**, 399 (1976).
16. G.A. Junk, 'Organic pollutants in water,' Presented at ACS Symposium Series No. 214, American Chemical Society, Washington, DC (1987).
17. P.R. Musty and G. Nickless, *J. Chromatogr.,* **89**, 185 (1974).
18. K.M. Sundaram, S.Y. Szeto, and R. Hindle, *J. Chromatogr.,* **177**, 29 (1979).
19. G.L. Lebel, D.T. Williams, G. Griffith, and F.M. Benoit, *J. Assoc. Off. Anal. Chem.,* **62**, 241 (1979).
20. I. Liska, *J. Chromatogr. A*, **885**, 3 (2000).
21. J.N. Little and G.J. Fallick, *J. Chromatogr. Sci.,* **16**, 517 (1978).
22. E. Chladek and R. S. Marana, *J. Chromatogr. Sci.,* **22**, 313 (1984).
23. A. Bacaloni, G. Goretti, A. Lagana, B.M. Petronio, and M. Rotatori, *Anal. Chem.,* **52**, 2033 (1980).
24. A. Di Corcia, C. Crescenzi, E. Guerriero, and R. Samperi, *Environ. Sci. Technol.,* **31**, 1658 (1997).
25. C. Crescenzi, A. DiCorcia, and R. Samperi, *Quad. Ist. Rec. Acque,* **112**, 7 (2000).
26. D.F. Hagen, C. Markell, F.L. DeRoos, and M.K. Bicking, *Organohalogen Compd.,* **2**, 137 (1990).
27. E.R. Brouwer, D.J. Van Iperen, D.J. Liska, H. Lingeman, and U.A. Th. Brinkman, *Int. J. Environ. Anal. Chem.,* **47**, 257 (1992).
28. D. Barcelo, G. Durand, V. Bouvot, and M. Nielen, *Environ. Sci. Technol.,* **27**, 271 (1993).
29. W.C. Koskinen and B.L. Barber, *J. Environ. Qual.,* **26**, 558 (1997).
30. H.A. Moye, M.R. Marshall, and W. Merlino, in 'Book of Abstracts, 214th ACS National Meeting, Las Vegas, NV, September 7–11, 1997,' AGRO-099, American Chemical Society, Washington, DC (1997).
31. O. Kicuchi and K. Saito, *Bunseki Kagaku*, **49**, 575 (2000).
32. J.N. Little and G.J. Fallick, *J. Chromatogr.,* **112**, 389 (1975).
33. I. Liska, E.R. Brouwer, A.G. Ostheimer, H. Lingeman, U.A. Th. Brinkman, R.B. Geerdink, and W.H. Mulder, *Int. J. Environ. Anal. Chem.,* **47**, 267 (1992).
34. E.R. Brouwer, H. Lingeman, and U.A.Th. Brinkman, *Chromatographia,* **29**, 415 (1990).
35. G. R. vander Hoff, R.A. Baumann, U.A.Th. Brinkman, and P. van Zoonen, *J. Chromatogr.,* **644**, 367 (1993).
36. S. Chiron and D. Barcelo, *J. Chromatogr.,* **645**, 125 (1993).
37. S. Chiron, S. Dupas, P. Scribe, and D. Barcelo, *J. Chromatogr.,* **665**, 295 (1994).
38. S. Lacorte and D. Barcelo, *J. Chromatogr.,* **712**, 103 (1995).
39. J. Slobodnik, O. Oeztezkizan, H. Lingeman, and U.A.Th. Brinkman, *J. Chromatogr.,* **750**, 227 (1996).
40. C. Aguilar, I. Ferrer, F. Borrull, R.M. Marce, and D. Barcelo, *Anal. Chim. Acta*, **386**, 237 (1999).

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41. R. Koeber, C. Fleischer, F. Lanza, K.S. Boos, B. Sellergren, and D. Barcelo, *Anal. Chem.,* **73**, 2437 (2001).
42. O. Bobleter, *Chromatographia,* **43**, 444 (1996).
43. I.G. McWilliam, *Chromatographia,* **17**, 241 (1983).
44. J.J. Rook, *Water Treat. Exam.,* **23**, 234 (1974).
45. L.H. Keith, 'Identification and Analysis of Organic Pollutants in Water,' Ann Arbor Science, Ann Arbor, MI (1976).
46. H. Schauenburg, H. Schlitt, and H. Knoppel, 'Assessment of a Moving Belt Type HPLC-MS Interface with Respect to Its Use in Organic Water Pollution Analysis', EUR 7623, Commission of the European Communities, Brussels, p. 193 (1982).
47. D. Volmer, *GIT Fachz. Lab.,* **38**, 19 (1994).
48. R.M. Caprioli, T. Fan, and J.S. Cottrell, *Anal. Chem.,* **58**, 2949 (1986).
49. P. Levi, *Analusis,* **16**, 224 717 (1989).
50. W.M. Muck and J.D. Henion, *Biomed. Environ. Mass Spectrom.,* **19**, 37 (1990).
51. T. Cairns, *Food Test. Anal.,* **3**, 15 (1997).
52. J.A. Sphon, *J. Assoc. Off. Anal. Chem.,* **61**, 1247 (1978).
53. U.S. Federal Register, 40 CFR Appendix B to Part 136, 'Definition and Procedure for the Determination of the Method Detection Limit – Revision 1.11' (1994).
54. J.-Y. Hu, T. Takako, and Y. Magara, *Water Res.,* **33**, 417 (1999).
55. E. Wickremesinhe, J. Brisbin, and J. Ruhl, in 'Book of Abstracts, 220th ACS National Meeting, Washington, DC, August 20–24, 2000,' AGRO-170, American Chemical Society, Washington, DC (2000).
56. B.A. Ingelse, R.C. J. van Dam, R.J. Vreeken, H.G. J. Mol, and O.M. Steijger, *J. Chromatogr.,* **918**, 67 (2001).

Sampling and analysis of soil

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1 Introduction

Field studies designed to investigate agrochemical behavior in soil are conducted for a variety of reasons. For example, these studies may be performed to understand soil, climatic, and use-pattern effects on the biological efficacy of soil-applied agrochemicals. This information is used to establish application rates as a function of soil texture and organic matter content, or to establish safe rotation intervals for succeeding crops. However, these studies are most often conducted to determine the fate of an agrochemical in the soil environment.

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Soil represents a significant environmental 'sink' for agrochemicals when they are applied, for example, as pre-emergence herbicide applications, fungicide coatings on seeds, and 'drench' insecticide treatments around the foundations of homes and buildings. Incidental agrochemical depositions onto soil result from the over-spray and wash-off of foliar pesticide applications and, to a minor extent, deposition of atmospheric residues. As a result of direct applications and indirect depositions on to soil, information is needed on the persistence and mobility of agrochemicals and their degradates in soil.

Field dissipation studies conducted on bare soil are the primary means by which soil dissipation rates of agrochemicals, and rates of formation and decline of their associated degradates, are determined under actual conditions. These studies are used to validate results of laboratory studies (e.g., metabolism and rate of degradation in soil and sediment, aqueous and soil photolysis, hydrolysis, sorption, and column leaching) that are used in formulating conceptual environmental dissipation models for agrochemicals. In addition, the propensity of an agrochemical to leach below the root zone can be assessed when these studies are properly conducted. More definitive studies, such as small-scale prospective groundwater studies and/or lysimeter

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studies, are necessary to establish fully the leaching behavior of a potentially mobile compound. Ultimately, the dissipation and mobility parameters derived from these field soil dissipation studies are used by regulatory and environmental scientists to predict environmental concentrations and fates of agrochemicals in different soil and climatic environments using computer simulation models. The purpose of this article is to present field and laboratory best practices that have proven useful in the successful conduct of field soil dissipation studies. Although focusing specifically on field soil dissipation studies, the soil sampling, sample processing, analytical, and curve-fitting techniques described herein pertain to other more general situations requiring the careful sampling and analysis of soils for agrochemicals.

The four main phases involved in a field soil dissipation study are (I) planning and design phase, (II) field-conduct phase, (III) sample processing/analysis phase, and (IV) data handling/reporting phase. Each phase is vitally linked to the next and each is critical to study success. Results from an otherwise perfectly executed study may be made useless by uneven test substance application or improper sampling, sample handling, and/or analytical techniques. Each of these phases is discussed below.

2 Phase I: field study design and logistics

Before the first soil sample is collected, several important, inter-related factors must be considered to ensure study success. These factors include the specific physicochemical properties of the agrochemical, its use pattern, the anticipated environmental conditions during use, and several practical considerations related to the analysis of soil (Figure 1). They determine the type(s) of soil to be investigated, agrochemical application method and rate, depth and frequency of sampling, and total amount of soil to be collected. Additional information, such as how the cores will be shipped, stored, sectioned and processed, and whether supplemental irrigation, if any, should be applied during the study must also be determined as part of the study design phase. These are all important details that should not be left to chance or addressed as an afterthought once the study is under way. Because each phase of study conduct is significantly impacted by the chemical and physical properties of the agrochemical, we begin with a review of key physicochemical properties affecting field study design.

2.1 Physicochemical properties

2.1.1 Anticipated persistence of parent molecule

Among the first issues to address in study design is how often soil samples should be collected and how long the study should be conducted. The frequency and duration of soil sample collection depend on the anticipated soil persistence of the agrochemical based on results from laboratory and/or other field studies. Because dissipation data are subjected to regression analysis and mathematical modeling in order to calculate a dissipation half-life ($T_{1/2}$) or DT_{50} value [unlike the $T_{1/2}$ value that is based on first-order kinetics, *DT* values make no assumptions as to the appropriateness of first-order kinetics in fitting dissipation data; see Phase IV (Section 5) for more details], the sampling regime must result in decline data that bracket the anticipated $T_{1/2}$ or

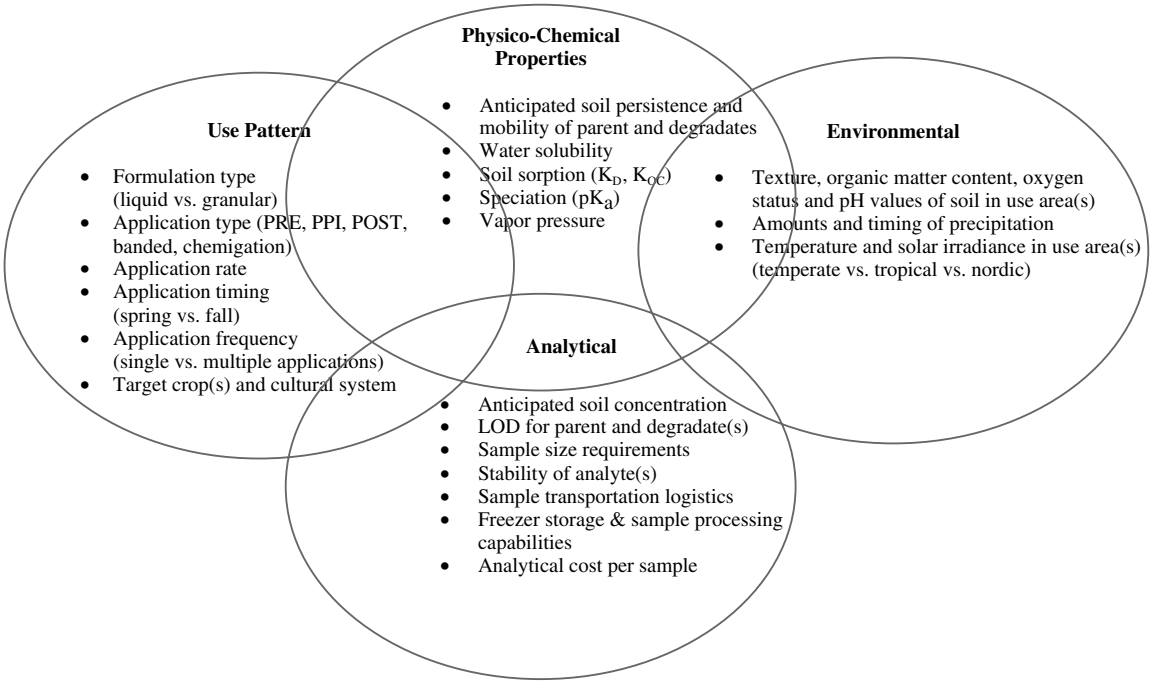

Physico-Chemical Properties
Anticipated soil persistence and mobility of parent and degradates
Water solubility
Soil sorption (K_D, K_{OC})
Speciation (pK_a)
Vapor pressure
Use Pattern
Formulation type (liquid vs. granular)
Application type (PRE, PPI, POST, banded, chemigation)
Application rate
Application timing (spring vs. fall)
Application frequency (single vs. multiple applications)
Target crop(s) and cultural system
Environmental
Texture, organic matter content, oxygen status and pH values of soil in use area(s)
Amounts and timing of precipitation
Temperature and solar irradiance in use area(s) (temperate vs. tropical vs. nordic)
Analytical
Anticipated soil concentration
LOD for parent and degradate(s)
Sample size requirements
Stability of analyte(s)
Sample transportation logistics
Freezer storage & sample processing capabilities
Analytical cost per sample

Figure 1 Inter-related factors affecting the design of terrestrial field soil dissipation studies

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DT_{50} value. The sampling points should occur at regular, evenly spaced intervals with four to five sampling points prior to the $T_{1/2}$ or DT_{50} value. If the data are heavily skewed towards the ends of a regression line, with few sampling points in between, erroneous estimates of the dissipation rate will occur.

For example, Figure 2 shows how the number and spacing of sampling points can affect $T_{1/2}$ determination. In Figure 2(a), a total of 15 sampling periods were fitted using a first-order model, resulting in an estimated $T_{1/2}$ of 10 days and a coefficient of determination (R^2) of 0.90. Here, six evenly spaced sampling periods occurred before the half-life and four immediately after the half-life. In Figure 2(b), two sampling periods prior to the half-life and five intermediate sampling periods were omitted from the calculations, resulting in a $T_{1/2}$ of 28 days. This value is almost three times longer than that estimated using the complete data set, and demonstrates how sampling frequency and spacing affect dissipation rate estimation.

When an environmental fate profile of an agrochemical and its degradates is not established, it is prudent to collect soil cores at frequent intervals, recognizing that it may not be necessary to analyze all of the contingency samples. A standard practice in field protocol design is to build 20% more sampling periods into the sampling regime than is thought necessary to adequately characterize the dissipation profiles. If only laboratory-derived soil persistence data are available, a general guide is to assume a 3–5 times greater degradation rate in the field than was observed in the laboratory. This empirical factor reflects that dissipation rates are often greater in the field as compared with laboratory data.[1]

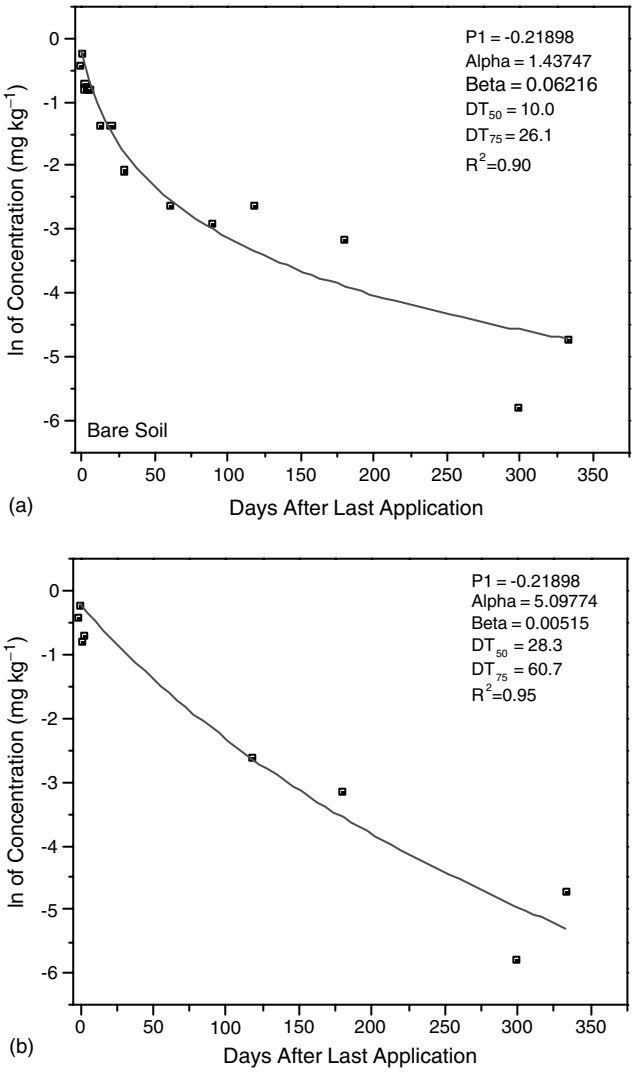


Figure 2 Influence of sampling frequency on first-order model parameters

Faster degradation in the field results from increased opportunities for volatilization, leaching, photolysis, and 'aging'. Moreover, microbial activities and biomass in soil may be significantly affected by techniques used to store, dry and fortify soils used in laboratory studies.[2–4] Thus, if a compound has a laboratory $T_{1/2}$ of 100 days, assume a $T_{1/2}$ of 20–30 days when designing the field sampling protocol. If a wide range of persistence is exhibited under controlled conditions, it is prudent to design the protocol to accommodate a range in dissipation rates (e.g., frequent early sampling coupled with prolonged sampling).

Vapor pressure (VP), water solubility (S_W), and soil sorption coefficients (K_{OC}) are key properties that govern volatilization of agrochemicals from soil.[5] Volatile compounds such as *S*-ethyl dipropylthiocarbamate (EPTC) (VP $\approx$ 4.5 Pa,

$S_W = 375\,mg\,L^{-1}$, $K_{OC} \approx 223\,mL\,g^{-1}$) and vernolate (VP ≈ 1.4 Pa, $S_W = 108$ mg L^{-1}, $K_{OC} \approx 260\,mL\,g^{-1}$) are readily lost from moist soil. In fact, their volatility makes it imperative to soil-incorporate these herbicides immediately after application to reduce volatilization. Frequent initial sampling is required when investigating volatile compounds such as EPTC and vernolate. This is especially true for the first 24 h when volatilization losses are often the greatest from moist soils. Intermediate volatilization losses have been observed for compounds such as trifluralin (VP ≈ 0.015 Pa, $S_W = 0.3\,mg\,L^{-1}$, $K_{OC} \approx 7200\,mL\,g^{-1}$) and fonofos (VP ≈ 0.045 Pa, $S_W = 17\,mg\,L^{-1}$, $K_{OC} \approx 1920\,mL\,g^{-1}$).[5] Volatility is not a major consideration in sampling protocols designed for compounds such as atrazine (VP $\approx 9 \times 10^{-4}$ Pa, $S_W = 33\,mg\,L^{-1}$, $K_{OC} \approx 147\,mL\,g^{-1}$) and prometon (VP ≈ 0.011 Pa, $S_W = 720$ mg L^{-1}, $K_{OC} \approx 95\,mL\,g^{-1}$).

Designing a sampling protocol to describe adequately the formation and decline of degradates requires prior knowledge of a compound's dissipation behavior. Degradates included in analytical protocols for field dissipation studies must first be identified in laboratory hydrolysis, soil/aqueous photolysis and soil/sediment metabolism studies. They usually must comprise $\geq 10\%$ of the applied dose in at least one laboratory study to warrant inclusion in a field dissipation study. However, degradates of known toxicological concern must be included, even when present at $<10\%$ of the applied dose. In some cases, minor transformation products are included to better describe the degradation pathway or to avoid 'parent only' dissipation profiles. Because of the increased analytical sensitivity they afford, radiolabeled test materials are sometimes used to track the formation and decline of degradates under field conditions.[6,7]

The appearance of degradates in field soil can be sporadic and transient in nature, making it difficult to obtain useful kinetic information under field situations. As a result, sampling protocol design becomes more complicated when the parent molecule and degradation product(s) exhibit widely different soil persistence. For example, although chlorsulfuron has a $T_{1/2}$ of 18 days, samples were collected for 540 days after last application so that the dissipation profiles of three primary degradates could be discerned.[8] Even with frequent and prolonged sampling, additional laboratory studies may be required to fully establish the dissipation kinetics of degradates whose dissipation profiles are not adequately captured under field conditions.

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2.1.2 Anticipated mobility in soil profile

The anticipated mobility of an agrochemical affects protocol design by determining maximum depth of sample collection. Properties governing soil mobility include S_w, acid dissociation constant (pK_a), soil sorption coefficients (K_D, K_{OC}) and VP.[9] The soil sorption of ionizable compounds is affected by pH and organic matter and clay content.[10] Weak organic acids and bases are less retained as soil pH increases due to increasing (weak acids) or decreasing (weak bases) ionization. Depending on their persistence, compounds with $S_W > 30\,mg\,L^{-1}$, $K_D < 5$ and/or $K_{OC} < 300\,mL\,g^{-1}$ are generally more likely to move below the root zone than sparingly soluble, strongly bound compounds such as trifluralin ($S_W < 1\,mg\,L^{-1}$, $K_{OC} > 5000\,mL\,g^{-1}$, $\log K_{OW} > 3$).[11] Thus, deeper soil coring is required for potentially mobile compounds compared with those which are less mobile. Ultimately, this

information is used to assess the maximum depth of leaching of the agrochemical under field conditions.

When the potential mobility of an agrochemical and/or its degradates is not known, it is prudent to collect sub-surface cores to a depth of 90–120 cm, recognizing that only certain sections of the cores may ultimately be processed and analyzed. For studies conducted for regulatory purposes, cores must be collected such that at least one core section is found to be free of quantifiable residues. As a result, full-length cores (90–120 cm) are almost always collected for studies conducted for ultimate submission to regulatory agencies, regardless of the anticipated mobility of the agrochemical. Additional information on the physicochemical properties of agrochemicals can be found elsewhere.[9,11,12]

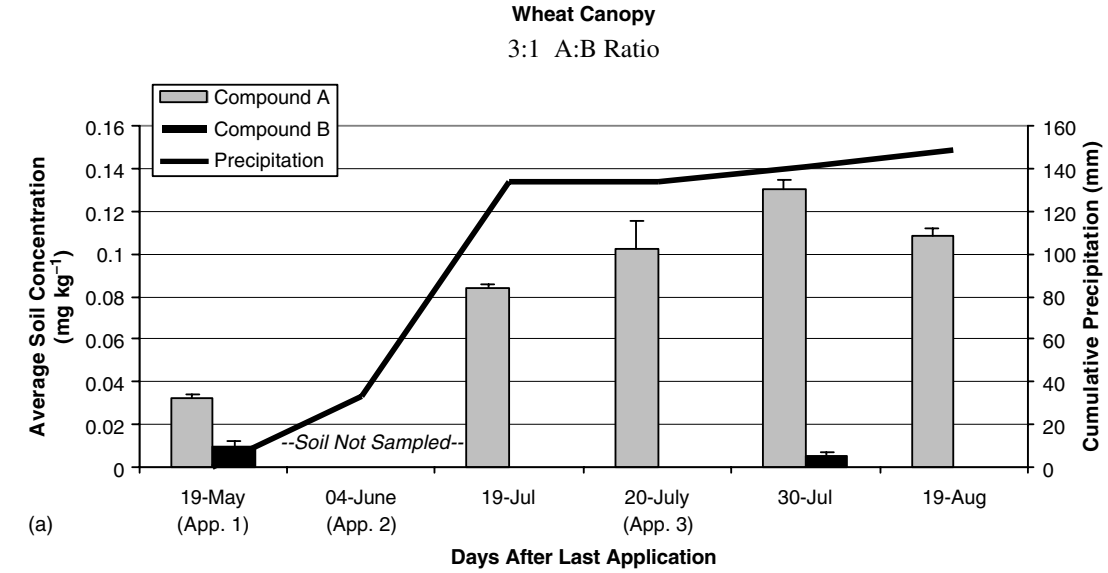
2.2 Use-pattern considerations

A guiding principle in the design of a field soil dissipation study is that study conduct should closely follow agricultural practices associated with the particular use pattern of the agrochemical being investigated. This requires knowledge of regional agricultural production practices for the targeted crop and an appreciation of how physical formulation, method of application, and soil and climatic factors affect agrochemical dissipation in soil. As discussed below, the ideal of closely following realistic agricultural practices has to be carefully balanced with practical considerations and the ultimate purpose of the study.

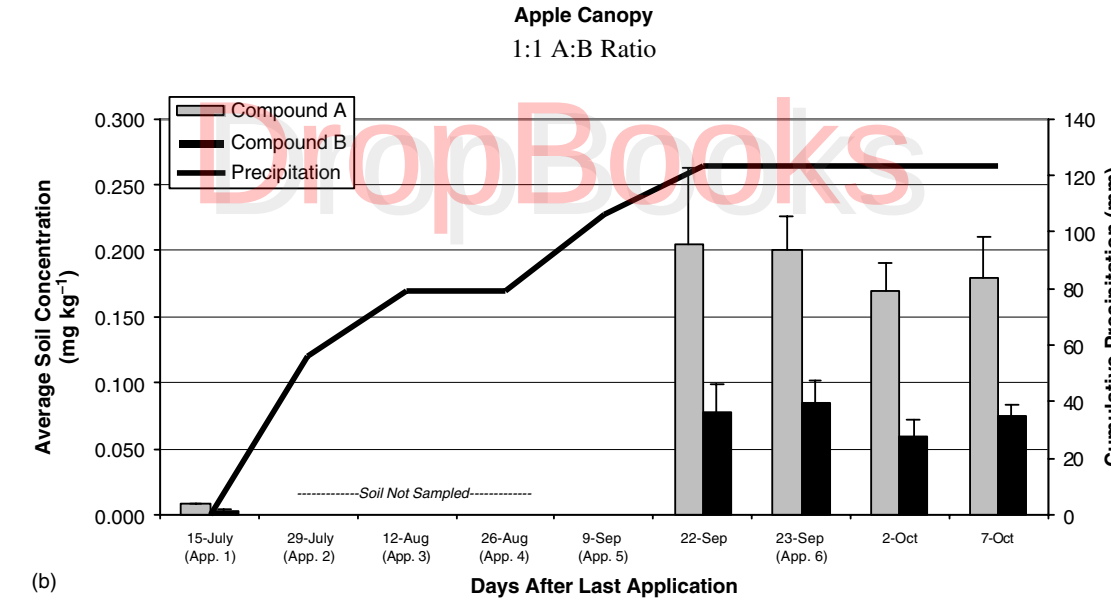
2.2.1 Bare-soil versus cropped studies

Determining whether to study the dissipation of an agrochemical in the presence or absence of a crop represents an important consideration in field study design. Bare-soil studies are useful in establishing soil dissipation kinetics for agrochemicals at their labeled rates but will omit potential rhizosphere, leaf photolysis, canopy shading, crop uptake and transpiration effects on dissipation and mobility. Cropped studies are useful in determining maximum plateau concentrations under actual-use conditions but cannot always be relied upon to yield results with the resolution necessary for accurate determination of soil dissipation kinetics. Moreover, the presence of an overhanging canopy, rocks, roots, and/or hardpans common to certain cropping situations (e.g., orchards) may impede deep soil sampling necessary for maximum depth of leaching determinations.

An increasingly important agronomic use pattern is reduced or no-till conservation practices where crop residues are allowed to accumulate on soil surfaces to reduce soil erosion and improve soil tilth. The effects that accumulated crop residues and concomitant changes in soil properties associated with conservation tillage have had on herbicide dissipation, for example, have been mixed,[13] but represent another important use-pattern consideration for certain agrochemicals. Because soil characteristics such as organic carbon content, pH, and microbial biomass require several years to be affected by conservation tillage practices, field sites must be carefully selected when the effect of crop residues on agrochemical dissipation is to be determined. Clearly, tillage would not be appropriate for no-till investigations.



Standard error bar shown about mean of three replications representing a composite of 10 soil samples/rep./sample period.



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Standard error bar shown about mean of three replications representing a composite of 10 soil samples/rep./sampling period.

Figure 3 Soil deposition of two agrochemicals after application to (a) wheat and (b) apple canopies

Figure 3 illustrates the difficulties that can arise when investigating agrochemical dissipation in soil following application to foliage. The figure shows the initial soil deposition of two agrochemicals that were applied as a mixture to field-grown wheat or apple trees. Three sequential applications of a formulation with a 3 : 1 ratio of

Compound A to Compound B were made to a mature wheat canopy while six sequential applications of a formulation with a 1 : 1 ratio of Compound A to Compound B were applied to mature apple trees. These studies closely mimicked actual agrochemical use patterns in terms of application rate and timing, application volume, irrigation, and other key agricultural practices.

When applied to wheat, Compound A was readily detected in soil samples collected beneath the treated canopy. Compound B was detected only sporadically in soil. Due in part to a greater application volume (1000 vs 300 L ha^{-1} water) and a higher application rate for Compound B, both compounds were more consistently detected in soil following application to apple foliage. It is often difficult to establish dissipation kinetics under these conditions because as residues in the soil dissipate, additional compound may continue to be deposited on the soil, resulting in a complex, variable dissipation pattern. As a result, it is not always practical or advisable to study soil dissipation in the presence of a crop.

A compromise to the 'bare-soil vs cropped studies' dilemma is to establish a bare-soil study in close proximity to the target crop. Using the above scenarios, a bare-soil study could be established by removing the aboveground portion of the crop wheat or the vegetation existing between the rows of trees in the apple orchard. The bare surface allows direct application of the agrochemical to soil, eliminating the effects of delayed and variable agrochemical deposition on soil commonly associated with foliar applications while exposing the agrochemical to edaphic and hydrogeological conditions that approximate those of soils underlying agronomic or horticultural crops.

For compounds applied to annual crops, another approach is to apply the compound to bare soil prior to crop emergence and follow the soil dissipation of the compound as the crop emerges and grows throughout its normal growing season.[14–16] This is the appropriate use pattern for pre-emergence compounds and represents another approach that may used to study the soil dissipation of foliar-applied compounds.

In terms of leaching potential, mobility is often considered 'worst case' under bare-soil conditions because the absence of crops eliminates transpiration losses, a major avenue of water loss from cropped soils.[17] Elimination of transpiration losses results in less upward movement of agrochemicals in the soil profile that, in turn, increases the potential for agrochemicals to leach over time. Leaching may also be greater under reduced-tillage conservation systems, largely due to increased numbers of intact soil macropores as compared with tilled soils (Ref. 13 and references cited therein). Moreover, use patterns where the compound is injected via sub-surface drip irrigation increase opportunities for the compound to leach and require additional design and sampling considerations.[18] Ultimately, the primary purpose of the study and the use pattern of the agrochemical dictate whether to use a bare-soil or cropped study design. Many of the technical considerations and techniques presented in this article pertain to both study types.

2.2.2 Region of use

Soil, climatic, and hydrogeological conditions vary widely between geographical regions. This variation occurs on a variety of scales and significantly affects agrochemical dissipation rates and fates in the environment. As a result, differences in

soil properties and the range of climates where a compound is used must be carefully considered. These factors also determine the number of study locations required to establish the full range of agrochemical dissipation behavior in field soil. A geographic information system (GIS) has been developed to identify comparable field study areas in the USA and Canada.[19]

Soil properties affecting dissipation include texture (sand, silt, and clay contents), clay type, organic carbon content and type, iron and aluminum oxide contents, porosity, overall fertility, moisture and oxygen status, temperature, pH, salinity, and microbial community structure and activity. Climatic factors for consideration include seasonal timing and amounts of precipitation, solar irradiance, and maximum/minimum temperatures that affect crop production. Field sites that accentuate leaching (low soil sorption capacity, high rainfall, high pH for weak organic acids) and/or persistence (high soil sorption capacity, low soil moisture, high or low soil temperature, high or low pH) help to define 'worst case' behavior for agrochemicals.

2.2.3 Supplemental irrigation

Crops may be grown under rainfall-fed or irrigated production systems. For a study designed to mimic an upland use pattern, the ability to irrigate test plots is required in the event that weather conditions turn drier than normal. Typically, 110% of the long-term monthly mean precipitation is applied to ensure that leaching opportunities for the agrochemical exist under study conditions. Water inputs for irrigated crops are determined using a soil-water budget method. The soil-water budget method is described in more detail in Section 3.3.9. In addition, irrigation is sometimes required to facilitate soil sampling in dense, hard-packed soils.[20]

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Whatever the case, the ability to irrigate test plots is an important consideration during field site selection. Sprinkler irrigation is preferred. Flood and furrow irrigation should be avoided since they may disturb surface residues, resulting in uneven residue distribution and/or inadvertent agrochemical loss from the study plots. Recommended irrigation practices are discussed in more detail in Section 3.3.8.

2.2.4 Soil incorporation of agrochemical residues

Certain soil-applied agrochemicals are incorporated into the soil after application to facilitate better contact with target organisms and/or to reduce losses due to photolysis and volatilization. Soil incorporation depths typically range from 2 to 10 cm. Incorporation is accomplished using a power rotary tiller, rolling cultivator, rotary hoe, disk harrow, or similar implement.[6,21,22] To maintain realistic study conditions, agrochemicals that are typically incorporated during use should be incorporated during field soil dissipation investigations.

Soil incorporation must be done with care to avoid the introduction of significant variability in agrochemical residues in soil. Studies have found that as much as a 50-fold variation in agrochemical residues may arise when incorporation is incomplete or uneven.[14,23] A single incorporation pass is not sufficient to mix agrochemicals thoroughly and, in practice, two or more passes are often necessary.[23,24] Provisions for soil incorporation must be made prior to study initiation and should follow good agricultural practices.

2.2.5 Application timing

The time of year in which a pesticide is applied significantly affects its dissipation rate due to temperature, moisture, and solar-irradiance effects on abiotic and biotic dissipation processes. For example, dissipation rates for agrochemical applications made in the springtime are normally greater than those observed for fall (autumn) applications.[8,15,21] Thus, the timing of agrochemical applications made in field soil dissipation studies should closely match those occurring under actual-use conditions.

2.2.6 Method of application

Agrochemicals are applied using a number of techniques. The method of application depends upon formulation type and the particular setting in which the chemicals are used. Granular formulations may be applied aerially or applied broadcast or banded using ground equipment.[21] Liquid solutions may be applied aerially or applied broadcast using ground equipment or by air-blast or chemigation.[16,18] Banded and chemigation applications require specialized application equipment and additional study design considerations to ensure that soil residues are sampled in a representative manner. Use patterns that involve agrochemical applications under plastic mulch affect dissipation and require access to specialized equipment used in plot establishment and test-substance application.[25] The most common application method used in field soil dissipation is broadcast application using a hand-held or vehicle-mounted spray boom. Proper broadcast application techniques are discussed in Section 3.

Another application-related factor affecting study design is the quantity of test material that is available for study. When applied as a commercial formulation, test-substance availability is generally not an issue, and relatively large, replicated areas can be treated with commercial application equipment. In contrast, radio-labeled test materials or agrochemicals being investigated early in the discovery process are applied in small amounts using small-plot techniques such as described by van Wesenbeeck *et al.*[6] and Zabik *et al.*[7] These small-quantity materials are usually prepared in formulation blanks to approximate the physicochemical properties of commercial products.

2.2.7 Application rate and frequency

Application rate is generally dictated by the labeled, or anticipated, application rate relevant to the particular use pattern being investigated. To improve analytical detection or to compensate for potentially low zero-time application recoveries, application rates are sometimes increased to 110% of the labeled application rate. An application rate greater than this level would be subject to regulatory scrutiny and may affect the dissipation rates of certain agrochemicals owing to potential short-term effects on sensitive soil microflora.

For low-use rate compounds applied on a grams per hectare basis, it has sometimes been necessary to apply the cumulative seasonal rate in a single application in order to improve analytical detection. Advances in analytical chemistry have greatly improved the trace-level detection of agrochemicals in soil but it is still prudent to verify that sufficient analytical sensitivity exists to detect agrochemicals at their anticipated soil

concentrations and, thus, allow dissipation rate determinations over time. Calculation of anticipated soil concentration is discussed in Section 2.3.1.

The proper frequency of applications made during a field study is a source of contention among environmental scientists. For example, some believe that the number should be determined strictly by the agrochemical's use pattern. For example, if the compound is typically applied four times over a growing season at 2-week intervals at a rate of 0.1 kg a.i. ha^{-1} per application (a.i. = active ingredient), it should be applied in this manner for a soil dissipation study. However, other scientists have found that dissipation data resulting from sequential agrochemical applications can be difficult to interpret, especially for degradates.[26] They would argue that the above compound should be applied in a single application of 0.40 kg a.i. ha^{-1}. For studies conducted for regulatory purposes, it is recommended that the application rate and frequency represent the 'worst-case' scenario in terms of agrochemical persistence and mobility.[27] A single application made at the maximum-labeled rate is often viewed as worst case in this regard. This latter approach also reduces costs associated with multiple applications, travel to field site, and application verification.

2.2.8 Application volume

The volume of spray solution in which an agrochemical is applied to a given area is relevant to nongranular formulations. Broadcast application volumes typically range from 200 to 600 L ha^{-1}. In an attempt to improve zero-time recoveries of agrochemicals, some practitioners diverge from actual-use conditions in terms of application volume and/or the number of passes made over the test plots during test substance application. This practice is based on the assumption that improved coverage of the soil surface results in more uniform agrochemical residues in soil samples. These practices deviate from standard agricultural practices but may be necessary to ensure a uniform application.

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2.3 Analytical considerations

2.3.1 Anticipated soil concentration and analytical sensitivity

As mentioned previously, one must ensure that sufficient analytical sensitivity exists to analyze the agrochemical at its anticipated soil concentration. In order to make this determination, one calculates the nominal zero-time soil concentration and compares this value to the limit of quantitation (LOQ) determined for the soil analysis method. Because the dissipation of agrochemicals in soil typically follows a biphasic or 'hockey stick-shaped' pattern (biphasic or hockey stick-shaped dissipation curves are characterized by initial rapid dissipation rates followed by substantially slower decline rates, resulting in soil residues that persist at low levels for a period of time[28]), regulatory agencies often require that the time required for an agrochemical to dissipate to 25% or 10% of the initial soil concentration (e.g., DT_{75} or DT_{90} value) be determined in addition to the DT_{50} value. Hence analytical methods are often developed to quantify residues equivalent to $\leq$5% of the initial applied mass so that DT_{90} values may be readily obtained. For relatively high use-rate compounds applied at kilograms per hectare rates, this generally does not pose a problem. However, for low use-rate

compounds applied at gram per hectare rates, the analytical sensitivity necessary for these low levels cannot be assumed and must be verified prior to study initiation.

For example, the expected zero-time soil concentration (C_0) of a compound applied at a rate of 2.2 kg a.i. ha^{-1} would be calculated by dividing the application rate (mg a.i. ha^{-1}) by the total weight of a 15-cm depth of soil. Assuming a soil bulk density of 1500 kg m^{-3}, the total weight of a 15-cm layer of soil is 2.24×10^6 kg ha^{-1}:

$$(2.2 \times 10^6 \text{ mg a.i. ha}^{-1})/(2.24 \times 10^6 \text{ kg soil}) \approx 1.0 \text{ mg a.i. kg}^{-1} \quad (1)$$

Similarly, the expected C_0 of a 0.168 kg a.i. ha^{-1} application rate would be:

$$(1.68 \times 10^5 \text{ mg a.i. ha}^{-1})/(2.24 \times 10^6 \text{ kg soil}) \approx 0.08 \text{ mg a.i. kg}^{-1} \quad (2)$$

The LOQ value necessary to follow residue decline to 5% of the initial value, as is typically needed for DT_{90} determination, would be 0.05 mg kg^{-1} for the higher application rate and 0.004 mg a.i. kg^{-1} for the lower rate. As a result, an LOQ of 0.01 mg a.i. kg^{-1} would be sufficient for the 2.2 kg a.i. ha^{-1} application rate but not for a rate of 0.168 kg a.i. ha^{-1}.

There are several approaches that might be taken to address the issue of insufficient analytical sensitivity indicated by the above calculations. For example, the LOQ might be lowered by increasing the total amount of soil extracted, reducing the volume of the final extract solution, improving method cleanup procedures to reduce the effects of interferences, and/or switching to a more sensitive method of detection. A brief overview of analytical techniques used for soils, with an emphasis on liquid chromatography/mass spectrometry (LC/MS) techniques, is given in Section 4.

Another approach to improving agrochemical detection is to apply more of the active ingredient to increase the initial soil concentration. As mentioned previously, however, one must be careful not to exceed greatly the labeled application rate of the compound as questions may arise as to concentration effects on the observed dissipation. A more common and acceptable approach is to section the upper soil core into smaller depth increments, yielding increased residue concentrations as the total amount of soil mixed with the residues decreases in each processed sample (Table 1).

Table 1 Anticipated zero-time concentrations (mg kg^{-1}) as a function of soil core length

	Core section length (cm)				
	0–15	0–10	0–7.5	0–5	0–2.5
Total soil weight (kg)[a]	2.24×10^6	1.49×10^6	1.12×10^6	7.47×10^5	3.73×10^5
Concentration estimate (mg kg^{-1})[b]	0.08	0.11	0.15	0.23	0.45

[a] Total soil weight per given depth per hectare; assumes a bulk density of 1500 kg soil m^{-3}.
[b] Calculations are based on a nominal application rate of 0.168 kg a.i. ha^{-1}. Soil core sectioning techniques are discussed in Section 3.

For example, if one must estimate a DT_{90} value given an application rate of 0.168 kg a.i. ha^{-1} and an LOQ of 0.01 mg a.i. kg^{-1}, one could further section a 0–15-cm upper core into 5-cm lengths, resulting in an increased ability to detect to 0.011 mg kg^{-1} as required by the LOQ:

$$C_0 \times 0.05 = 0.23 \text{ mg a.i. kg}^{-1} \times 0.05 = 0.011 \text{ mg kg}^{-1} \approx \text{LOQ} \quad (3)$$

Regardless of how the upper core is ultimately sectioned, the 15–120-cm depth cores are typically sectioned in 10–15-cm lengths for analysis. Techniques used to section soil cores are presented in Section 3.3.6.

2.3.2 Agrochemical residue variability and sample number requirements

Variability exists in every aspect of study conduct and must be carefully controlled for meaningful field soil dissipation results. Variability under the investigator's control includes that associated with soil surface preparation, agrochemical application, soil incorporation (if any), sample collection, sample processing, and sample analysis.[29] Variations in the biological, chemical and physical processes affecting agrochemical dissipation in soil can be large within a field, and are responsible for the observed increases in variability with time.[30–34]

The blocking techniques designed to statistically minimize effects of soil heterogeneity require prior knowledge of soil texture, fertility, and/or other gradients that occur across the test site.[35] Such information is not typically known when field soil dissipation studies are being established, and the positioning of study plots is usually based instead on matching plot dimensions with those of the test site. As a result, the impact of soil heterogeneity may only be partially minimized through careful visual assessments of soil conditions made during site selection (see Section 3.1) and collection of ample numbers of soil cores. The greater the variability in soil residue levels, the greater is the number of samples required to estimate the dissipation rate of an agrochemical.

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The need for additional samples to compensate for soil heterogeneity must be reconciled with labor, storage, transportation, analytical, and other constraints that add significantly to study costs. Satisfactory results have been obtained from numerous field studies using three or four treated replications with 5–10 soil cores collected from each replication per sampling period.[6,7,15,20] These replication/repetition numbers strike a reasonable balance between the need for samples sufficient in number to characterize agrochemical dissipation versus financial and logistical constraints associated with sample collection and analysis.

2.3.3 Sample homogenization

In practice, the number of soil samples that is actually analyzed is reduced by the preparation of composite samples. Here, multiple samples from a given replication and sampling period are blended together to yield one composite sample for analysis. Composite samples are statistically justifiable as they increase the precision with

which the mean residue concentration in soil can be estimated while decreasing the total number of samples analyzed.[36,37]

Careful attention must be paid to the homogenization of soil samples because incomplete or careless blending may result in significant variability among agrochemical residues and may place in jeopardy an otherwise well-executed study. Clayey soils are generally more difficult to homogenize than sandy or loamy soils and, therefore, often require additional processing time. Thorough homogenization also becomes increasingly more difficult with increasing soil moisture. Soil homogenization techniques are discussed in more detail in Section 4.1.

2.4 Basic experimental designs for field soil dissipation studies

At this stage in planning, the essential study design information listed below should be determined and a written study plan (i.e., protocol) including these key study details prepared. A formal, pre-approved study plan is required for field soil dissipation studies conducted under Good Laboratory Practice (GLP). A written study plan for non-GLP studies is highly recommended since the document serves as valuable guidance for study personnel.

Study design consideration	*Basis for design*
Number and locations of test sites and required soil properties	Region(s) of test substance use and use pattern
Cropped vs bare-soil surface	Use pattern and study purpose
Small vs large plot	Test material availability
Application type	Relevant use pattern and physical formulation
Application rate, frequency, and timing; need for soil incorporation	Relevant use pattern
Sampling frequency, duration, and depth	Anticipated persistence and mobility of agrochemical and degradates
Number of replications and repetitions	Anticipated variability in soil residues and cost constraints
Amount of soil to collect and core sectioning	Depends upon specific analytical procedures (and associated LOQ) and available sample storage and processing capabilities
Supplemental irrigation (sprinkler)	Necessary for most dryland and irrigated cropping scenarios

Additional information regarding applicator-boom width, spray-tank capacity, and the wheelbase of any vehicle-mounted soil sampling equipment used during the study is also required to ensure that the field plot design accommodates size restrictions of field equipment.

2.4.1 Control plot

Untreated (control) soil is collected to determine the presence of substances that may interfere with the measurement of target analytes. Control soil is also necessary for analytical recovery determinations made using laboratory-fortified samples. Thus, basic field study design divides the test area into one or more treated plots and an untreated control plot. Unlike the treated plots, the untreated control is typically not replicated but must be sufficiently large to provide soil for characterization, analytical method validation, and quality control. To prevent spray drift on to the control area and other potential forms of contamination, the control area is positioned $\geq$15 m away and upwind of the treated plot, relative to prevailing wind patterns.

2.4.2 Treated plots

Factors used to determine treated plot size include the (1) available quantity of test substance, (2) total number of samples to be collected, (3) specific space requirements of soil sampling equipment, (4) foot and equipment traffic within and between plots necessary for plot establishment/maintenance and sample collection, and (5) necessity for minimizing preferential flow to subsoil through sample boreholes. The treated test area must be large enough to provide the required number of soil core samples and ensure that human activities do not affect or influence the dissipation of the agrochemical. The study design should always allow for extra 'contingency' samples beyond the anticipated level of sampling deemed sufficient at the time of study design. Once the total number of samples to be collected has been determined, the availability of necessary study supplies (e.g., plastic probe liners, caps, labels, bags, etc.) and freezer storage capacity should be determined.

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2.4.3 Small-plot designs

A wooden or metal containment box surrounding the treated area is commonly used when a small quantity of test material is to be applied.[6,7] The box, typically rectangular in shape and partially buried beneath the soil surface, serves to isolate the treated area from surrounding soil and protect against wind and water erosion. A one- to two-nozzle application boom that moves along guy wires or tracks is often used to ensure even application. Radiolabeled materials having two or more label positions often serve as replicates in these studies.

2.4.4 Large-plot designs

The need to collect soil samples repetitively with time while minimizing soil surface disturbance associated with foot and equipment traffic precludes the completely random collection of soil samples from study plots. One of the most common field designs, used successfully in numerous field dissipation studies, divides the treated test area into three or four blocks (i.e., replicates). The blocks are further subdivided into subplots as shown in Figure 4. The number of subplots is dictated by the number of sampling dates plus a 20% contingency since soil samples are taken only once from each subplot using this design.

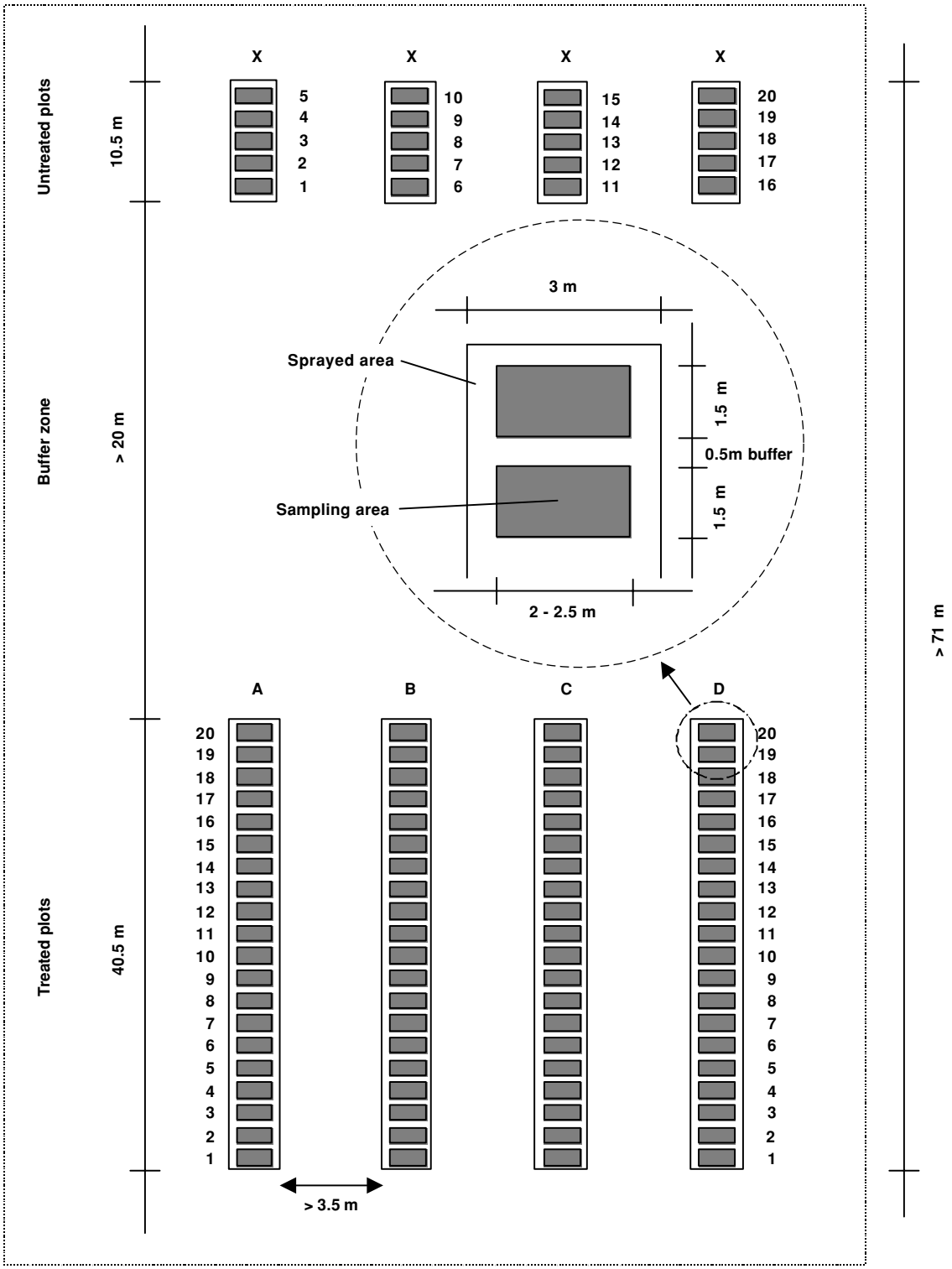


Figure 4 Randomized block design using four replications having 20 sub-plots each

The width of an individual treated replicate should not be wider than 3 m to enable test substance application using a single pass of a conventional plot sprayer. The application is made in the same direction as the layout of the plot. If multiple boom widths are used in treating a single plot, it is critical that areas of potential over-spray and under-spray are avoided during soil sampling. Study designs requiring multiple application passes within a single treated area are not recommended owing to potential issues arising from areas of over- or under-spray.

Five soil cores are typically collected from a predetermined subplot within each replication at each sampling period. As mentioned previously, the number of soil cores collected increases with increasing residue variability. The order of subplot sampling is determined using a randomization procedure[38] or by random-number subroutines common to many computer spreadsheet programs. The areas between the treated replicates serve as buffer zones and provide access lanes for study personnel and vehicles. Within each row, the subplots are separated by a buffer zone of 0.5 m. An important advantage of the completely randomized block design is that sample collection is distributed across the entire test plot, helping to capture effects of soil spatial variability on agrochemical dissipation. The design presented in Figure 4 is readily adapted to bare-soil and cropped studies.

Additional planning and sample numbers are often required when agrochemicals are applied as banded rather than broadcast applications. The soil sampling techniques devised for banded fertilizer applications provide a good basis for the sampling of agrochemical residues.[39,40] For example, the recommended approach for sampling fields receiving banded nitrogen fertilizer applications involves the collection of 15–30 composite cores taken between the banded rows and inter-rows of the field.[39] Sampling at multiple positions perpendicular to the application band provides a measure of agrochemical distribution throughout the surface soil. Similarly, determining representative soil sampling locations for agrochemicals applied by chemigation is not a trivial undertaking and requires increased sample numbers to account for increased residue variability.[18]

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2.4.5 Plot markers

A field soil dissipation study usually lasts between 1 and 2 years; long-term soil accumulation studies may last for up to 6 years. Hence, it is essential that test plots are clearly marked to ensure accurate sampling for the duration of the study. Durable, highly visible markers (stakes) made of plastic, metal, or wood should be located at the main corners of the treated and control plots. Additional markers indicating replication and subplot number or line number, as appropriate, must also be installed. Weather-proof signs must be installed that clearly indicate the Study Director and contact information, study number, test substance and application rate, and study initiation and termination dates. This information helps to prevent application and sampling errors. Plot markers and signs should be checked regularly to ensure that they are legible and in good physical condition.

Permanent markers outside the study area should also be located and used in the event that one or more plot markers are inadvertently moved or lost. One option is to locate a minimum of two permanent reference points outside of the study area

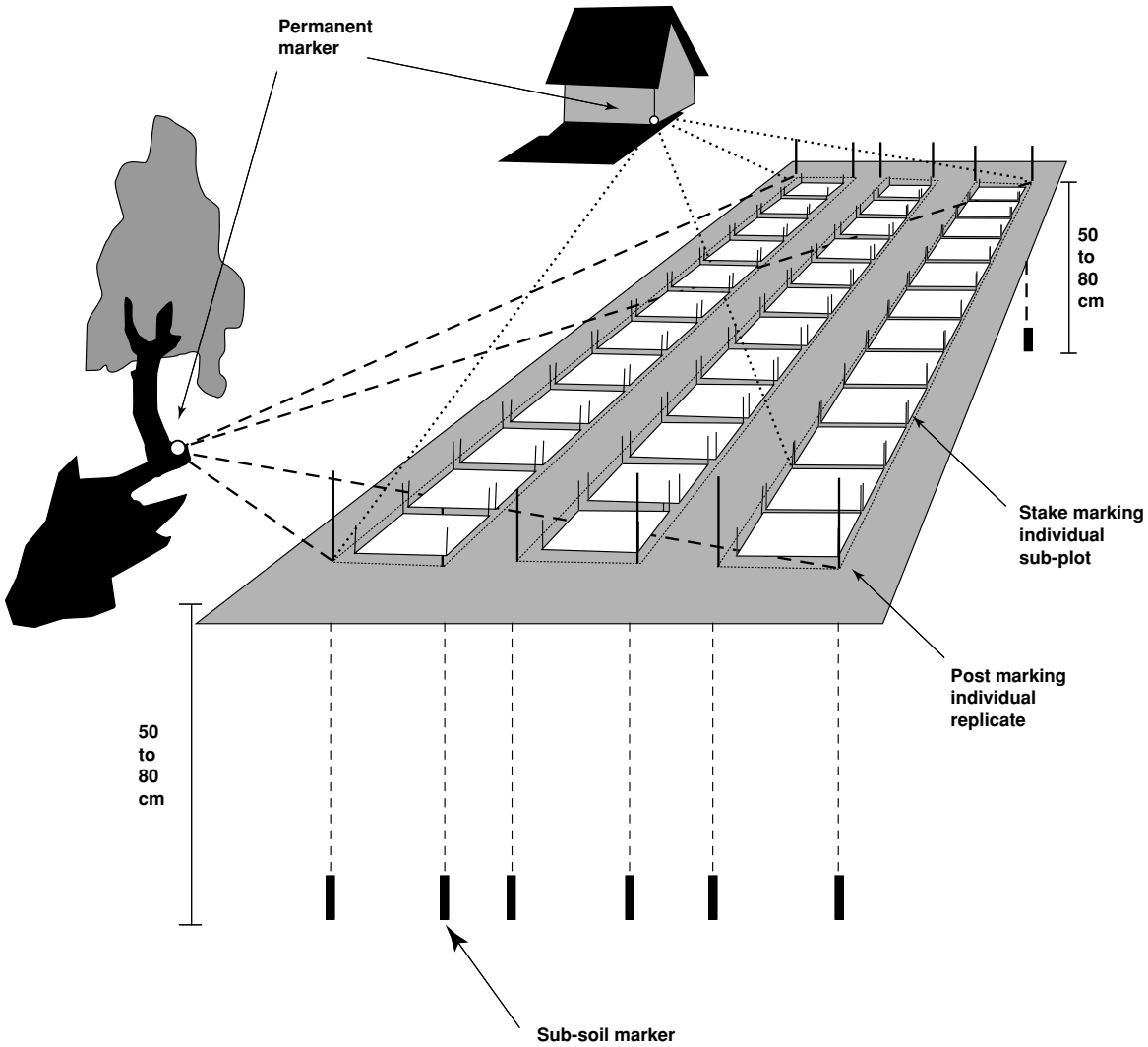


Figure 5 Techniques used to mark test plots in field soil dissipation trials

that can be used to re-survey the test area by triangulation (Figure 5). The distances to prominent points such as the ends of sampling plots should be recorded in the study records and indicated on corresponding plot maps. Another option is the use of sub-soil markers that are detected by induction (Figure 5). Because these markers are placed 60–80 cm directly below prominent points in the study area, it is unlikely that they will be moved during the study. The sub-soil markers are especially useful in long-term accumulation studies that involve seasonal plowing or cultivation and when permanent landmarks are not conveniently located near the study area.

2.5 Additional considerations

2.5.1 Study documentation

Overall study success depends upon the careful documentation of key aspects of study conduct. As mentioned previously, a formal, written study plan (protocol) is required for GLP studies and is highly recommended for non-GLP studies. Other key information to document in the study records includes example calculations involving the application rate, anticipated zero-time concentration, and those associated with the analysis of soil. Additional documentation should include the source, purity, test site location(s), soil textural class, diagrams of test site layout, type and inner diameters of soil corers, sampling depths, pertinent weather parameters, amount and timing of all supplemental irrigation, and the names of all personnel involved with study conduct. The date and time of each application, sample collection, freezer storage, sample extraction, and analysis should all be carefully recorded. Any events that result in deviations from the written protocol must be carefully recorded in the study records and, in the case of GLP studies, the Study Director notified of these events within 24 h of their occurrence. Photographs taken during test substance application and sampling and of the equipment related to these activities are useful in reconstructing key aspects of study conduct. Thorough documentation is as vital for non-GLP research as it is for studies conducted for regulatory purposes.

2.5.2 Safety

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Equipment used to apply agrochemicals and to collect and process soil is inherently dangerous. The appropriate personal protective equipment must be worn and minimally includes protective eyewear and gloves. Additional protective equipment may include spray suits, respirators, steel-toed boots, and hearing protection, depending on the particular materials being investigated and equipment being used. Large physical force is required to insert a soil probe into the ground; this same force can crush or amputate human limbs. Hence, workers must be well trained in the operation of sampling equipment. Fieldwork also requires physical exertion so caution should be observed when working in high temperature and humidity conditions. Studies involving the application of radiolabeled materials require prior written permission from the appropriate regulatory authorities as well as special provisions for the proper removal and disposal of treated soils and sub-soils.

3 Phase II: field study conduct

Each of the five main steps in field conduct (site selection, test plot layout, test substance application, sample collection, and sample storage/handling) is addressed below.

3.1 Test site selection

Once the targeted study regions, soil textures, space requirements, and other key aspects of study design have been determined, the search for suitable test sites

begins. Test site selection is critical to the success of a field soil dissipation study as field-related factors have a major influence on the overall outcome of the study. Even for bare-soil studies, an 'agriculturally viable' soil that would be capable of growing a healthy crop is usually desired. Hence it is important to ascertain the soil's recent cropping and management history before choosing a particular site.

Table 2 lists basic criteria that can be used during field site selection for bare-soil and cropped studies. Priority among the selection criteria depends upon the particular goals of the study but certain factors (e.g., slope $>1\%$, excessive rocks, flood prone, potential plot disturbance by wildlife) usually serve to exclude certain sites automatically. If the region of interest is far away, it is best to seek the assistance of university investigators, extension agents, and consultants who are familiar with the regional agricultural practices and local soil and climatic conditions.

Table 2 Site-selection criteria for field soil dissipation studies

Selection criterion	Priority[a]	Basis for selection	Comments
Region	A or B	Site must match the climatic, soil, and agricultural conditions typical of the target crop	Some crops are grown only in certain regions (e.g., rice) while others are common to many regions (e.g., maize). Thus, selection of a test region may be restrictive or relatively flexible
Soil properties	A	Soil texture (sand, silt, clay), organic matter/carbon content, and pH Stones, roots, and hardpans must be largely absent to allow representative sampling of soil profile Soil properties should appear uniform over test site	Soil texture data should be available at time of site selection. Soil properties must match study purpose. This can be 'realistic use' conditions, 'realistic worst-case' or 'worst-case' in terms of agrochemical mobility and persistence Must ensure that the majority of samples can be taken from the deepest sampling horizon. Information about sub-soils can be obtained from soil maps, test coring and on-site interviews
Site topography	Exclusion	Must have slope $\leq 1\%$ Site must not be susceptible to flooding Shallow water table or tile drains must not interfere with sampling	These are exclusion criteria that have to be carefully determined during on-site inspection Site must be level to prevent losses of agrochemical due to surface run-off and soil erosion Site must not be susceptible to runoff from other areas higher than test site
Size of test site	B	Depends on study design. The minimum area required for a typical large-plot design is about 0.25 ha	Test site must allow for test design plus sufficient buffer zone around perimeter of field to protect against external disturbance For bare-soil studies, shady sites should be avoided

(*Continued overleaf*)

Table 2 —*Continued*

Selection criterion	Priority[a]	Basis for selection	Comments
Cropping history and previous pesticide use	Exclusion	The cropping and pesticide history for the previous 3 years must be well documented The test substance must not have been applied to site within the past 3 years	This information is crucial and evidence of careful record keeping reflects favorably upon the future reliability of a field cooperator Prior application of agrochemical forming identical/similar degradation products as test substance should be considered as potential analytical interferences Previous management practices (e.g., soil amendments, tillage, crop type) should have been uniformly applied across test site
Irrigation	Exclusion	Site must be equipped with sprinkler irrigation	Irrigation is necessary to ensure 110% of historical rainfall for dryland settings or to follow regional irrigation practices in irrigated cropping settings
Test site security	A	Access of unauthorized persons, livestock, etc., must be restricted	Potential impact of any nearby construction, utility lines, rights-of-way, etc., must also be assessed
Plot maintenance	B	Expertise must be available to maintain the test site and, if cropped, to take care of the crop	For bare-soil studies, the soil surface must be carefully prepared prior to test substance application and kept weed-free without disturbing the test areas. If the test is cropped, the crop should be treated according to Good Agricultural Practice. In case of a soil accumulation study, the field may be cultivated and cropped each season for up to 6 years
Ownership	A	Access to test site must be guaranteed for the duration of study	Owner must agree to grant access to the site for duration of study plus possible time extensions. As a result, sub-leasing of the test site is not preferred. *This criterion is extremely important for long-term studies such as field soil accumulation studies*
Weather station/ weather data requirements	A	On-site weather station is preferred and may be mandatory for certain studies. Minimally, a station must be located within 10 km of test site	In certain cases, a weather station located within 10 km of the test site may be sufficient. If water balances are to be determined, an on-site weather station is necessary to measure, at a minimum, precipitation, solar radiation, wind speed, relative humidity, and air temperature

[a] 'Exclusion' implies that criteria must be fulfilled without compromise since the study may be jeopardized if the criteria are not met; 'Priority A' implies some flexibility after careful consideration; 'Priority B' factors offer the greatest flexibility in terms of site selection.

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3.1.1 Collection of control soil

Once test sites have been identified, control soil should be collected and returned to the laboratory. This soil is used to (1) verify soil texture and related properties, (2) ensure adequate analytical recovery of target analytes, and (3) determine the presence of potential background interferences in the soil.

3.1.2 Soil surface preparation

Preparation of the soil surface is critical to achieving acceptable results with minimal variability. Surface roughness due to the presence of crop debris or soil clods makes representative sampling nearly impossible. This same material also interferes with sample homogenization. As a result, the importance of proper soil surface preparation for bare-soil studies cannot be overstated. If vegetation exists on the selected site, it must be removed for bare-soil study designs. Vegetation can be removed by application of a nonselective herbicide such as glyphosate, paraquat, or glufosinate followed by mowing, raking, and harrowing once the vegetation has died.

A combination of techniques is normally required to smooth the soil properly. For example, disking is usually followed by multiple passes of a rolling-cage cultivator. If necessary, individual subplots can be hand-raked. Sandy soils are the easiest to prepare and dry quickly after rainfall. Silt loam to clay loam soils form clods when worked too wet. Hence timing field preparation around rainfall and soil moisture content is always a factor in preparing test plots. Heavy clay soils containing >40% clay pose real challenges in terms of surface preparation owing to excessive clod formation and surface cracking and should be avoided. When clayey soils are investigated, increased numbers of soil samples should be collected to compensate for the additional variability typically associated with these soils.

In addition to being smooth, it is preferable that the soil surface be firmly packed. This is because loose soil is not always retained in large-diameter sampling probes. Firming of the soil surface may be accomplished using a turf roller or equivalent. Alternatively, the soil surface may be prepared in advance of study initiation to allow rainfall or irrigation to settle and firm the soil. This latter approach also allows soil surface depressions to be observed and avoided when laying out the test plots.

3.2 Test substance application

Accurate and even application of test substance is absolutely critical to study success. If the application is highly variable or deviates significantly from the target application rate, the study results may be technically unusable and/or unacceptable to regulatory authorities. Accurate agrochemical application begins with careful calibration of the spray equipment. Hence Study Directors should be familiar with sprayer calibration techniques,[41,42] even if they will not be personally making the applications.

Braverman *et al.*[43] found that factors responsible for inaccurate pesticide applications made for crop residue trials (i.e., application rates applied at >10% or <5% of the target rate) were improper boom height (60% of errors), miscalculation of application rate (26% of errors), and variations in pass time (14% of errors). Application rate calculations must be carefully performed and double-checked, preferably by a second individual. Calculations involving products containing more than one test substance can be particularly confusing and the application rate for each active ingredient must be clearly stated in the field protocol. Similarly, one must clearly distinguish application rates based on active ingredient versus acid equivalents for agrochemicals prepared in various salt formulations. For a given salt formulation, an application rate based on acid equivalents will always be more than that based on active ingredient. For more details on calculating application rates, see Anderson.[41]

Spray nozzle type plays an important role in the success of agrochemical application. For broadcast applications to soil, flat fan nozzles should be used. Newer spray tips such as the DG TeeJet, XR TeeJet, Turbo TeeJet and similar nozzles supplied by Lechler and Hardy have provided acceptable results in a number of studies. For a given nozzle type, the lower the application pressure, the larger is the spray droplet size and the less potential for spray drift. Similarly, the closer the boom is positioned to the soil surface, the less is the potential for spray drift.[44] Most applications are made with spray tips having 80° or 110° spray angles and boom heights of about 50 cm above the soil surface.

Wind speed is another important factor affecting applications. Because modern analytical techniques used in soil analysis are capable of detecting slight differences in residue concentrations, experienced applicators are cautious with regard to wind effects on pesticide drift. A hand-held anemometer should be used to measure wind speed at spray-boom height prior to and during test substance application. Applications should not occur when wind speeds exceed 3 m s^{-1}.[44] In regions where excessively windy conditions are the norm, it may be necessary to build wind blocks to protect the test plots during application. Wooden frames covered in plastic or fiberglass sheeting have been successfully used for this purpose.

3.2.1 Application verification

A combination of techniques is typically used to verify the accuracy and precision of agrochemical applications to soil. For example, the catch-back method or pass-time method is typically used in conjunction with analytical results from application verification monitors to confirm proper application. The catch-back method involves measuring the spray solution volume before and after application to double check that the desired volume of test solution was actually applied to the test plots. Experienced applicators are often able to apply within ±2% of the targeted spray volume.

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The pass-time method involves measuring the time that it takes the applicator to pass over a test plot of known length and comparing this time to the speed used in calculation. For example, a typical walking speed for an applicator carrying a hand-held boom is about 1.3 m s^{-1}. At this speed, it would take about 30 s to apply an agrochemical along the 40.5-m length of one replication (i.e., block) depicted in Figure 4. An actual pass time of 31 s would suggest that about 103% of the target application rate was applied to the test plot. As with spray volume, experienced applicators are often able to apply within ±2% of the targeted pass time. Field protocols typically require that the application be within ±5% of the target spray volume or pass-time value; pass-times or spray volumes greatly exceeding these criteria should be closely scrutinized and may warrant termination of the study.

Application verification (AV) monitors are devices that are placed within test plots to measure actual spray deposition that occurred during application. The main function of AV monitors is to show whether or not the intended amount of test material was actually deposited on the soil surface. Application monitors consisting of soil-filled containers, paper disks, polyurethane foam plugs, and glass Petri dishes have all been used successfully for this purpose. Prior to using a monitor in the field, it is important to determine that the test substance can indeed be successfully extracted from the monitor and that the compound will be stable on the monitor under field conditions.[45]

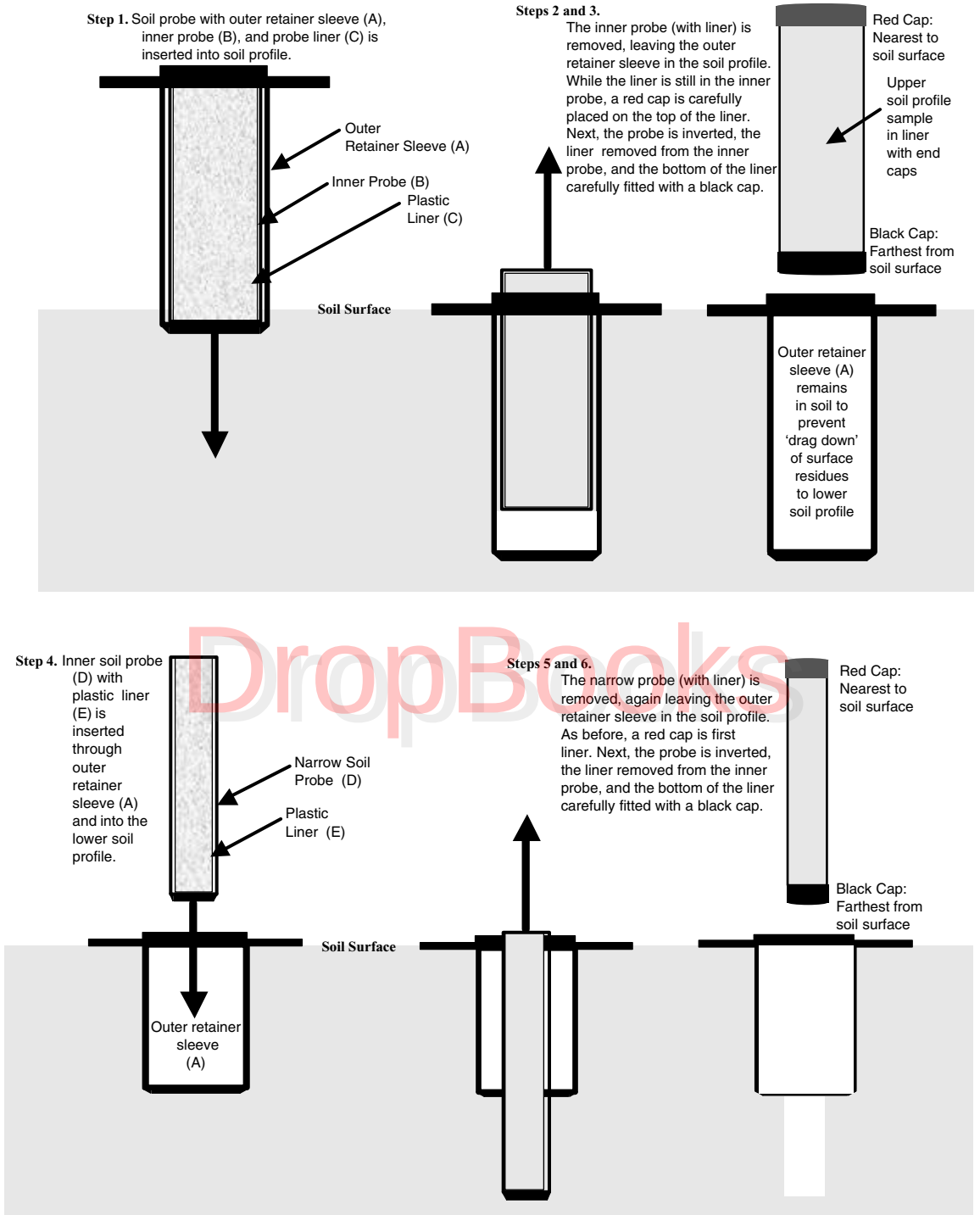
Application monitors are positioned in pre-determined locations shortly before test substance application. Immediately after application, the monitors are collected and stored in a freezer until they are extracted and analyzed. Soil samples must not be collected from locations previously covered by the monitors. Hence the monitors should be placed only in the unsampled buffer zones between the subplots. Alternatively, their positions within the test plots can be clearly marked using plastic flags or stakes and these locations not used for soil collection. The main advantages of using AV monitors rather than zero-time soil cores to verify application rates are that delays between application and zero-time sampling are greatly reduced (important for labile materials), and errors often associated with soil sampling are avoided.[29]

3.3 Soil sampling techniques

Over the years, many soil collection techniques have been developed and tested to determine their suitability for field dissipation studies. The biggest challenge confronting researchers is to collect representative samples from various depths of soil. Of particular concern is how to collect samples from the lower soil profile when a highly concentrated agrochemical residue layer exists at the soil surface immediately after application. Depending on the environmental fate properties of the compound, this challenge may exist for some time after application. The following provides an overview of sampling techniques that have proven useful in addressing these concerns in field soil dissipation studies.

The artificial downward movement of agrochemical residues caused by soil sampling is commonly referred to as drag down. Several coring techniques using similar overall approaches have been devised to prevent drag down and cross-contamination of soil samples. In the method depicted in Figure 6, a probe (e.g., 5.7×15 cm) with an associated outer sleeve is inserted into the soil. Once the probe and soil core have been removed, the outer retaining sleeve is left in the ground to ensure that the resulting borehole does not collapse, thereby preventing the contamination of lower soil depths by surface residues. Next, a smaller diameter probe (e.g., 3.8×120 cm) is inserted through the hole kept open by the outer retainer sleeve and forced down to collect soil lower in the profile. (Note that the lower probe length typically exceeds the length of the desired core length to offset less-than-full cores that occur commonly under field conditions, i.e., a 120-cm probe is used to ensure that 100-cm cores are collected.) Both probes are designed for use with a plastic liner. During sampling, it is the plastic liner that actually receives the soil so that soil does not touch the steel tubes except at the cutting tip.

As the plastic liners are removed from the probe, they are capped on both ends, the appropriate labels affixed, and promptly placed in a freezer (an in-field sectioning technique used for further partitioning of the 0–15-cm core is described later in this section). By convention, red plastic caps are placed on top of the core (i.e., the end that was closest to the soil surface) and black caps are placed on the bottom. Use of the two-color capping system is important when the cores are sectioned at a later time. This approach is referred to as zero-contamination sampling and is the industry standard in field soil dissipation.


Step 1. Soil probe with outer retainer sleeve (A), inner probe (B), and probe liner (C) is inserted into soil profile.
Outer Retainer Sleeve (A)
Inner Probe (B)
Plastic Liner (C)
Soil Surface
Steps 2 and 3.
The inner probe (with liner) is removed, leaving the outer retainer sleeve in the soil profile. While the liner is still in the inner probe, a red cap is carefully placed on the top of the liner. Next, the probe is inverted, the liner removed from the inner probe, and the bottom of the liner carefully fitted with a black cap.
Red Cap: Nearest to soil surface
Upper soil profile sample in liner with end caps
Black Cap: Farthest from soil surface
Outer retainer sleeve (A) remains in soil to prevent 'drag down' of surface residues to lower soil profile
Step 4. Inner soil probe (D) with plastic liner (E) is inserted through outer retainer sleeve (A) and into the lower soil profile.
Narrow Soil Probe (D)
Plastic Liner (E)
Soil Surface
Outer retainer sleeve (A)
Steps 5 and 6.
The narrow probe (with liner) is removed, again leaving the outer retainer sleeve in the soil profile. As before, a red cap is first liner. Next, the probe is inverted, the liner removed from the inner probe, and the bottom of the liner carefully fitted with a black cap.
Red Cap: Nearest to soil surface
Black Cap: Farthest from soil surface

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Figure 6 Diagram of zero-contamination soil sampling procedure

The insertion of a sampling probe into soil requires considerable force. As a result, collection of soil cores by human power is generally limited to the top 20–25 cm. Sampling below this depth requires some form of mechanical assistance. A common method used to insert and remove the probes is by hydraulic ram mounted on a three-point hitch of a tractor. A percussion method using an electric rotary hammer can also be used but is physically more demanding than hydraulic equipment and requires that the sampling tubes be removed from the ground via a ground jack. Pressing soil tubes into the ground is the least soil-disruptive technique that is currently used.

When a tractor for soil sampling is not available or it is logistically not feasible to transport such heavy sampling equipment to distant field sites, the coring approach depicted in Figure 7 represents another viable option. Using this method, the top 5 cm of soil is first carefully removed using a hand trowel from within a metal retainer sleeve and placed into a pre-labeled container. Next, a narrower probe that can be lengthened by attaching additional sections of pipe is used to collect discrete sub-soil samples. As before, plastic cartridges within the probe prevent the soil from actually contacting the metal probe. The probe can be inserted into the soil profile by electric drill, rotary hammer, or plastic mallet.

3.3.1 Influence of soil core diameter on study results

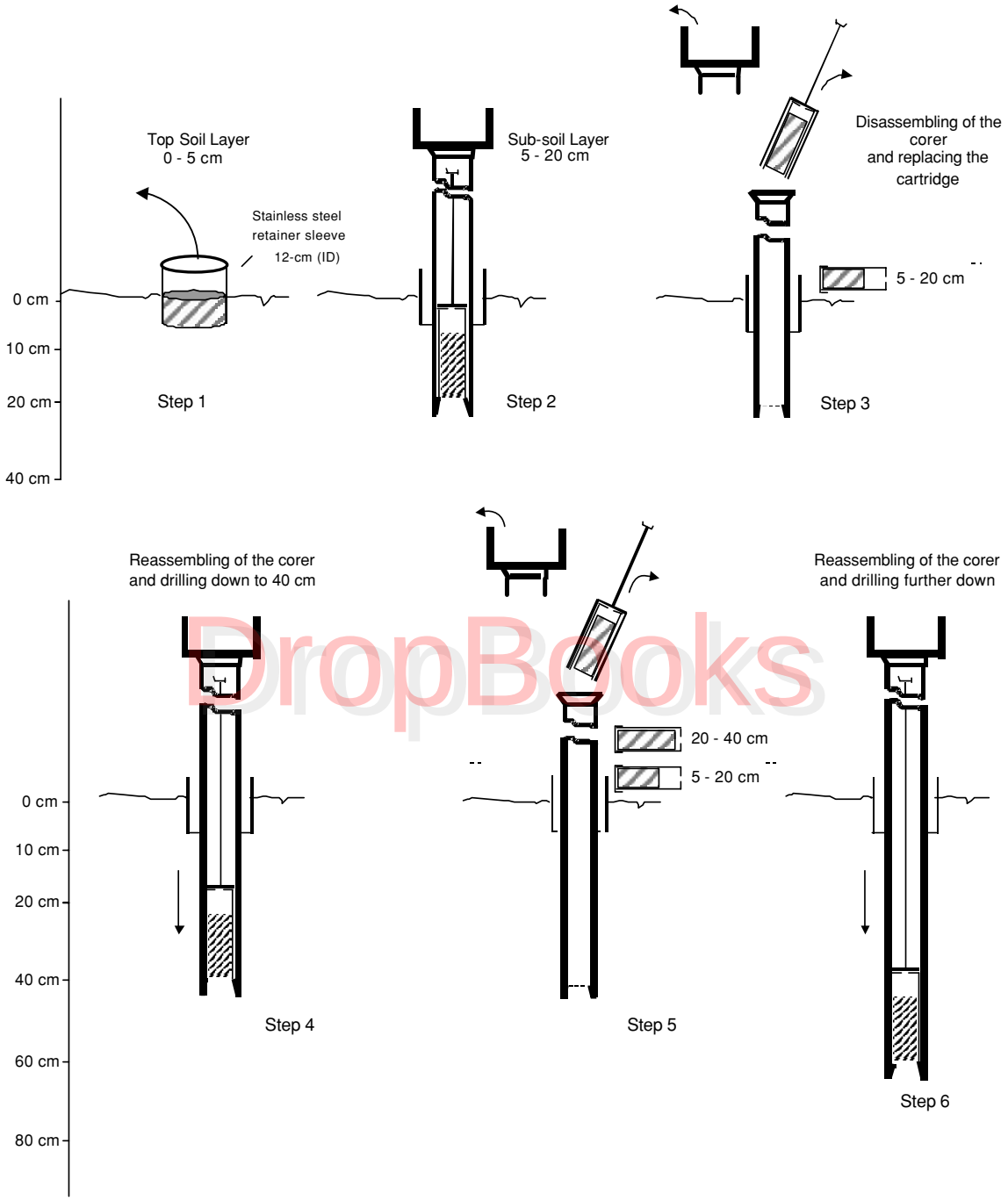
Assuming proper soil surface preparation (i.e., smooth with no soil clods or crop debris) and test substance application, the diameter of the soil probe does not generally impact observed pesticide residue concentrations in soil or associated variability.[14,30] Nevertheless, a minimum diameter of 5 cm for the upper soil probe is recommended to improve sampling under less than ideal conditions. Increasingly, researchers are using probes having diameters >5 cm with good results under a variety of field conditions.

3.3.2 Minimizing plot disturbance and cross-contamination

Great care should be taken while moving in and around the plots so that the sampling areas are not disturbed. The importance of minimizing soil surface disturbance and drag down during sampling is critical as one tries to assess the potential mobility of an agrochemical. This is particularly an issue when one attempts to collect many samples from a relatively small area. In general, the risk of sub-surface contamination is greatly minimized by using zero contamination sampling techniques.

To avoid cross-contamination of control samples, untreated controls are collected before the treated samples. Preferably, personnel who handle the upper cores should be different from those handling the lower depth cores. This further reduces potential cross-contamination of lower depth cores. Sampler handlers should change their gloves each time a new subplot is sampled. The use of disposable shoe covers also lessens the possibility of cross-contamination.

Once the soil cores have been collected, all boreholes must be backfilled with untreated soil (with frequent tamping) to prevent bypass flow that could transport residues into the lower soil profile. After backfilling, flags or stakes should be placed at the boreholes. This serves as an additional check to ensure that sub-plots are not sampled more than one time during the study. (Note that these boreholes should



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Figure 7 Alternative zero-contamination sampling method for soil

be periodically checked for subsidence over time and backfilled with soil again, if necessary, to prevent water infiltration.)

3.3.3 Cleaning procedure for soil sampling equipment

All sampling equipment coming in contact with treated soil (e.g., sample probes and sectioning equipment) must be thoroughly cleaned between compounds and collection periods. Cleaning is best accomplished by first brushing off any soil adhering to equipment. The next step is washing with pressurized water or soap and water, and finally rinsing with a solvent such as acetone or isopropyl alcohol, alone or in combination with clean water. The use of a solvent will facilitate faster drying of equipment.

3.3.4 Protection of sample integrity

All application verification and soil samples must be individually labeled with unique sample identification (ID) and other identifying information such as study ID, test substance name, sample depth, replicate, subplot and date of collection, as appropriate. Proper study documentation requires that sample lists and labels be created prior to work commencing in the field. Water- and tear-resistant labels should be used since standard paper labels may become water-soaked and easily torn during sample handling. Sample lists should have the same information on them as the labels and are a convenient place to record plot randomization, initials of the individual who collected the sample, and date of collection. As such, the sample list is important in establishing chain of custody from the point of sample collection until its arrival at the laboratory.

As soon as the sample has been properly labeled and recorded, it should be placed in a generator-powered chest freezer located directly in the field. A flat-bed trailer can be used to transport freezers to and from the field site. Insulated boxes filled with dry-ice can be used as a substitute for freezers. However, chest freezers typically work better than dry-ice since they allow more cold air circulation around the samples, facilitating more rapid freezing.

After the samples have been placed in the freezer, it is critical that they remain frozen until analysis. Electronic temperature data-loggers can be used to monitor conditions during storage. Simpler techniques, such as inverting plastic tubes partially filled with ice or placing plastic bags containing ice cubes, can also be used in combination with a mercury thermometer (any movement of the ice in the inverted tube or melting of the ice cubes indicates that the soil samples may have been subjected to temperatures $>0\,^{\circ}\mathrm{C}$ and, hence, sample integrity potentially compromised). Since electronic data-loggers are fairly inexpensive, however, continuous monitoring of freezer storage conditions is strongly recommended.

3.3.5 Zero-time recovery and importance of the soil micro-layer

Proper sample collection and handling are the key to acceptable agrochemical recovery at zero time. The zero-time sample interval is defined as the first sample collected after application. Zero-time soil samples should be collected within 3 h after application. Zero-time soil core concentrations, such as those given in Table 3,

Table 3 Summary of zero-time soil concentration and application verification (AV) monitor results for Pyraclostrobin applied at two field sites

Site location (state)	Nominal soil concentration (mg kg^{-1})	Calculated soil concentration based on average pass time (mg kg^{-1})	Maximum observed concentration on (mg kg^{-1})	Day maximum concentration observed (DALA)[a]	Recovery (%) based on application rate (0.28 kg a.i. ha^{-1})
(A) Zero-time soil recovery results					
CA – bare soil	0.25	0.281 ± 0.003	0.236	1	94 (104)[b]
FL – bare soil	0.25	0.282 ± 0.003	0.123	0	49 (53)[b]

[a] Days after last application.

[b] The number in parentheses denotes procedural correction using a 90% recovery for the CA site and a 93% recovery for the FL site.

	AV – fortified samples: mean concentration (μg)		AV – spray samples: total a.i. recovered (μg)		
Site/application no.	Expected fortification (nominal/assessed)	Observed fortification	Expected AV – spray	Observed AV – spray	Recovery (%)
(B) Application verification (AV) monitor results					
CA – App. 1	420.0/423.8	419.3	535	529.3	99
CA – App. 2		403.4	535	483.2	90
CA – App. 3		387.0	535	480.3	90
CA – App. 4		413.1	535	507.4	95
FL – App. 1	420.0/423.8	365.4	535	476.2	89
FL – App. 2		349.1	535	482.4	90
FL – App. 3		385.0	535	501.2	94
FL – App. 3		372.3	535	482.2	90

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are calculated by first subtracting any parent residue present in the core before last application (e.g., −T4) from the parent residue measured immediately after the last application (e.g., T4). For example, at the CA site, the soil concentration of BAS 500 F one day after last application (DALA) was 0.769 mg kg^{-1}. Prior to application, the soil concentration was 0.533 mg kg^{-1}. By subtraction, a concentration of 0.236 mg kg^{-1} was determined for BAS 500 F in the 0–8-cm section. This results in a zero-time soil recovery of 94% [(0.236 mg kg^{-1})/(0.25 mg kg^{-1}) × 100]. The parent residue concentration used to calculate recovery was the maximum concentration reached at any time during sampling after the last application. Zero-time core recoveries (corrected) ranged from 53 to 104% for the FL and CA sites (Table 3). These data show that even when considerable effort has been expended on proper test substance application (as evident by the excellent pass-time and AV recovery results) and sampling, zero-time recoveries are frequently lower and more variable than desired.

Discrepancies between AV monitor and pass-time (or catch-back) results and actual zero-time soil concentrations are most likely due to residue losses occurring during sample handling. Similar discrepancies may also arise for very labile compounds owing to rapid abiotic and/or biotic losses in soil; the presence of degradates in zero-time samples would indicate that low zero-time recovery was due to degradation losses. Immediately after application, all residues, with the exception of those compounds that are soil incorporated, are located in the uppermost layer of the soil core. This thin layer of surface soil is called the soil micro-layer. Loss of soil micro-layer residues is believed to be the main reason for low and/or highly variable zero-time recoveries from soil cores. Initial loss of the soil micro-layer is also believed to be the reason why maximum residue concentrations commonly occur days to weeks after application rather than at time-zero.[46] Until these surface residues are redistributed into the core by capillary action, precipitation, or irrigation, they remain subject to loss. Careful handling of the soil samples in the field and laboratory remains especially critical until surface residue redistribution has occurred.

Empirical evidence supporting the role of soil micro-layer losses in zero-time issues is given by the often-seen rise in post zero-time residue recoveries. The improved recoveries likely result from the micro-layer residue redistribution that reduces losses of the highly concentrated surface residues. There has been some speculation that zero-time core recoveries may be due to volatilization losses not measured by standard laboratory studies. If this were the case, however, increases in residue concentrations would not occur over time since volatilized residues would be lost to the atmosphere.[46]

3.3.6 Sectioning of soil cores

The upper soil core can be further sectioned into ≥2.5-cm lengths according to study needs and purposes. Sectioning of the upper core can be done in the laboratory but is most efficiently performed immediately after the core has been removed from the soil profile. In-field sectioning begins by using a metal or plastic 'punch' having a wide circular surface on one end to push the lower portion (i.e., the end furthest from soil surface) of the core out of the liner to the desired length. Next, a metal cutting tool (e.g., knife or spatula) is used to slice the soil core at the correct length. As the soil is being sliced, it is directed into a pre-labeled sample bag. This process is repeated, working from the lower to upper portion of the core, until all the appropriate sections

have been sliced away. The sample bags should be rotated in and out of the on-site freezer until all the sectioning depths have been collected from each core within a subplot. This technique works well for all soil textures.

Once the lower 15–120-cm cores are completely frozen, they can be further sectioned into 10–15-cm lengths using a hacksaw or band saw. As before, red and black caps are placed on the tops and bottoms of each newly created core section. Each new section also receives a unique sample ID number and new label containing all pertinent sample information. Care must be used when cutting frozen cores to prevent damage to original sample labels. An advantage of the sampling approach shown in Figure 7 is that the soil cores generally require no additional sectioning.

3.3.7 Field-fortification samples

In order to determine the dissipation rate and assess the potential mobility of an agrochemical in soil, it is crucial that the residue level measured in a particular sample reflects the actual concentration present in the soil profile at the time of sampling. If this basic assumption cannot be assured, the validity of resulting data may be questioned. Regulatory concerns have arisen over past improper sample-handling practices that might have artificially accelerated agrochemical dissipation in the soil samples. This could occur, for example, whenever samples are exposed to elevated temperatures and/or direct sunlight for extended periods of time prior to freezer storage. As a result, regulatory authorities have requested that a set of fortified samples having a known amount of active ingredient be prepared in the field. These field fortification samples are intended to indicate how well the integrity of the actual field samples was preserved during sample collection, transportation, and storage. If the field-fortified residues are found to be stable, the sample handling conditions are deemed sufficient also to have protected the integrity of the actual field samples. In contrast, if the recovery from the field fortification samples is low, this implies that sample integrity was compromised at some point during study conduct.

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Although theoretically sound, field fortification samples often generate as many questions as they answer. This is because accurate and precise fortification of soil is difficult to accomplish under field conditions except when the field site is very near the supporting laboratory. For a distant field site, the fortification solution is typically prepared and assayed in the laboratory prior to overnight shipment. If agrochemical recovery from the resulting field fortification samples is low, this may be due to accelerated dissipation, problems associated with the fortification solution itself or improper technique used by field personnel. Shipping fortifying solutions to the field is further complicated by the fact that many active ingredients make only suspensions, not true solutions. Once frozen or left without agitation for extended periods, these formulations are difficult to re-suspend, as is required for proper soil fortification. As a result, acceptable recovery from field spikes helps to address the issue of sample integrity, but poor recovery only results in more questions as to its cause.

A solution to this dilemma is to place soil samples immediately in a freezer located in the field, the temperature of which is continuously monitored, as described previously. Laboratory-prepared storage study samples can then be used to determine test substance stability under freezer storage conditions that match those used in the field and during transportation and final storage. If a valid laboratory storage stability

study indicates that residues are stable, any observed decline in soil residues can then be assumed to have occurred in situ. Details on the conduct of a freezer storage study are given in Section 4.

3.3.8 Test plot maintenance

The guiding principles in test plot maintenance are to (1) minimize soil surface disturbance at all times, (2) ensure that control and treated plots are similarly maintained, (3) avoid applying other agrochemicals that may interfere with sample analysis or that are otherwise contrary to the purpose of the study, (4) follow the prescribed irrigation policy determined for the study site, and (5) keep bare-soil test plots free of vegetation, as follows.

For bare-soil studies, vegetation is controlled on an 'as-needed basis' by application of nonselective herbicides (e.g., glyphosate, paraquat, glufosinate) or by careful hand weeding. Vegetation control may be required on a weekly basis during the growing season. The use of glyphosate or paraquat is a widely accepted means of controlling unwanted vegetation in and around test plots, and has the added advantage of controlling weeds without physically disturbing soil surfaces. Because physical disturbance of the soil surface is to be avoided, hoeing or other forms of mechanical removal should not be used in the actual test plots. Vegetation that is pulled by hand should remain on the test plots to avoid inadvertent removal of agrochemical residues.

3.3.9 Irrigation

Because soil moisture plays such a critical role in determining agrochemical dissipation rate and mobility, it is important to devise carefully an irrigation plan that clearly specifies the timing and amount of irrigation that is to be added at each study site. One must be able to justify all irrigation applications based upon the relevant agricultural practices in the study region and actual use pattern of the agrochemical.

For studies conducted in regions of irrigated agriculture, the plots must be irrigated according to the soil-water budget method. This is determined by calculating the evapotranspiration rate for the target crop (ET_c) and adjusting irrigation amounts to 110% of the ET_c:

$$ET_c = ET_0 \times K_c \tag{4}$$

$$\text{Irrigation to apply} = ET_c \times 110\% \tag{5}$$

where ET_0 is the actual daily evapotranspiration rate and K_c is the specific crop coefficient based on the targeted crop and appropriate growth stage. Deficiencies should be reconciled about every 10 days, as required.

In regions of rain-fed agriculture, the test plots must receive 110% of the monthly historical rainfall. Differences in this total should be reconciled every 10 days. If the plots do not receive 110% of historical monthly rainfall, the study may be severely compromised.

Apply the supplemental water inputs via sprinkler irrigation. Do not flood or furrow irrigate since these practices may disturb soil surface residues. Be aware that even

sprinkler irrigation can cause uneven application of water and, if leaks occur, severe erosion of the soil surface. Therefore, regularly inspect irrigation equipment and function. The control and treated plots must be irrigated in a similar manner. Record the volume and date of all irrigations, the source of irrigation water, and the type of irrigation system used. If water begins to pool or run off of the soil surface, stop irrigating immediately. Resume irrigation only after the risk of runoff is over. To avoid runoff, carefully match the application rate to the soil infiltration rate. Note that, in cold climates, irrigation equipment is winterized to prevent damage from freezing and is generally not available for use during the winter months.

4 Phase III: sample processing and analysis

Once soil samples have been received and properly logged in by the laboratory, there is a multi-step process required to isolate agrochemical residues from the sample matrix so that sensitive, reproducible analysis can occur. Residue methods for agrochemicals in soil involve the basic steps shown in Figure 8.

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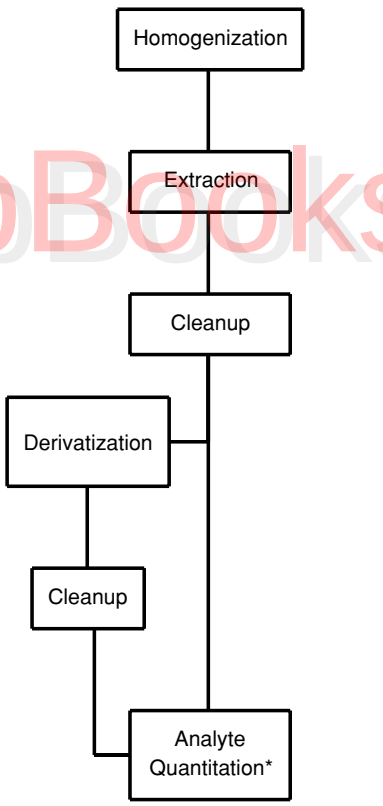


Figure 8 Schematic of general analytical method for soil analysis

A general overview of each of these steps is given below. This is followed by a specific example involving an increasingly powerful quantitation technique, liquid chromatography/tandem mass spectrometry (LC/MS/MS).

4.1 Sample homogenization

Soil homogenization is the critical first step in the analysis of soil samples. Improper homogenization can lead to variable results that seriously confound the interpretation of soil residue data. Samples are commonly homogenized using equipment called size-reducing mills. Size-reducing mills can be further categorized as being 'grinder', 'rotary blade', or 'hammer' type mills. Each of these has advantages and disadvantages but the ability to mix uniformly the anticipated volume of soil and the ease with which the mill can be cleaned are key considerations when choosing a particular mill. The design of the mill should also prevent the loss of fine soil particles generated during the blending process. Other key aspects of sample homogenization are addressed below.

4.1.1 Protecting sample integrity

When processing samples, they should always be milled using dry-ice in amounts sufficient to ensure that the samples remain frozen during homogenization. As discussed previously, protecting sample integrity is of utmost concern throughout every aspect of study conduct. The use of adequate dry-ice also helps keep soil from sticking to the mill. Some mills have been designed to use liquid nitrogen rather than dry-ice for cooling, and also work well with soils.

4.1.2 Minimizing cross-contamination

To minimize cross-contamination, soil cores are processed beginning with the lowest depth samples and progressing to the surface samples. It is very important that the mill be thoroughly cleaned between samples so as to minimize the risk of cross-contamination. The machinery should be thoroughly cleaned with water followed by a water–solvent solution such as acetone. Typically, the machine should be cleaned after running one replicate set of samples from the lowest depth to the surface. If the samples have coarse fragments in them, it may be necessary to sieve the samples prior to homogenization. As mentioned previously, soils with a large percentage of clods or rocks should be excluded during the site selection process since they also interfere with sample collection in the field.

4.1.3 Ensuring thorough sample homogenization

Before processing actual study samples, and periodically during the course of a study, it is important to test the thoroughness of the homogenization procedure using soils having a range of textures. This is typically done by measuring the analytical variance between sub-samples, and is the only reliable method for determining the effectiveness of a blending technique. Depending on the soil type and sample size, it may be necessary to pass the sample through a mill twice to ensure proper homogenization. For example, experience has shown that when using a rotary-blade type mill, two passes are normally required for proper homogenization of turf or sod samples. When

Table 4 Tepraloxydim analytical results used to determine efficacy of soil homogenization procedure

Description		Residue found (mg kg^{-1})
Sample weight = 10 g of soil		
Sample 1	Control	Not detected
Sample 2	Fortified sample, 0.1 mg kg^{-1}	0.101
Sample 3	Treated sample, replicate 1	0.120
Sample 3, duplicate analysis	Treated sample, replicate 1	0.110
Sample 4	Treated sample, replicate 2	0.050
Sample 4, duplicate analysis	Treated sample, replicate 2	0.057
Sample weight = 5 g of soil		
Sample 5	Control	Not detected
Sample 6	Fortified sample, 0.1 mg kg^{-1}	0.099
Sample 7	Treated sample, replicate 1	0.110
Sample 7, duplicate analysis	Treated sample, replicate 1	0.180
Sample 8	Treated sample, replicate 2	0.054
Sample 8, duplicate analysis	Treated sample, replicate 2	0.068
Sample weight = 2 g of soil		
Sample 9	Control	Not detected
Sample 10	Fortified sample, 0.1 mg kg^{-1}	0.102
Sample 11	Treated sample, replicate 1	0.148
Sample 11, duplicate analysis	Treated sample, replicate 1	0.133
Sample 12	Treated sample, replicate 2	0.059
Sample 12, duplicate analysis	Treated sample, replicate 2	0.063

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turf samples are being processed, it is also essential that the sod plug be totally frozen so that the plug will break up as it passes through the mill.

An example of adequate sample homogenization is given in Table 4. The experiment was conducted with two replicate treated soil samples. Each replicate was analyzed in duplicate. Three different sample aliquots (2, 5 and 10 g) were used from each replicate. Analyses of controls and fortified samples were also conducted concurrently with treated samples to evaluate method performance (i.e., extraction recoveries). These results show that residue values are the same regardless of sample size. Thus, thorough homogenization of soil samples coupled with rugged analytical methodology provides for satisfactory residue analysis.

4.2 Sample extraction

An efficient and reproducible extraction procedure is mandatory when analyzing agrochemicals in soil. An overview of common soil extraction techniques is given below.

4.2.1 Solvent selection

Soil samples are generally extracted with one or more organic solvents mixed with up to 10% (v/v) water. A wide variety of solvents is used for extraction, the choice

of which depends upon the polarity of the compound to be extracted.[47] For example, extraction with methanol and methanol–water usually works well for compounds with medium to high polarity. Acetonitrile is another common solvent used in soil extractions. Sometimes pH adjustment is also required for compounds that are acidic or basic in nature (e.g., ammonium carbonate is often added to improve the extractability of weak organic acids). Starch-encapsulated formulations may benefit from an enzymatic pretreatment prior to extraction from soil.[48]

Several extraction techniques are used in the analysis of soil. The following are brief descriptions of some of the most commonly used techniques.

4.2.2 Mechanical shaker

A commonly used extraction technique involves shaking soil with a suitable solvent on a mechanical shaker at about 300 rpm. After extraction, the soil extracts are collected by centrifugation followed by decantation or filtration. This technique could be used for any amount of soil samples (from 10 to >100 g). Soil samples greater than 100 g require efficient agitation to achieve acceptable recoveries.

4.2.3 Soxhlet extraction

This technique is used to extract effectively analytes that are polar in nature and strongly bound to soil. Typically, a solvent mixture containing a water-miscible solvent and an apolar solvent (e.g. methanol–dichloromethane) is used. A small aliquot of soil (10–30 g) is dried by mixing with sodium sulfate and refluxed for 8–16 h to extract the residues.

4.2.4 Sonication

This technique is used mainly for nonpolar compounds. Typically a small aliquot of soil (10–30 g) is dried by mixing with sodium sulfate prior to extraction. Next, the sample is extracted with a solvent for 10–20 min using a sonicator probe. The choice of solvent depends on the polarity of the parent compound. The ultrasonic power supply converts a 50/60-Hz voltage to high-frequency 20-kHz electric energy that is ultimately converted into mechanical vibrations. The vibrations are intensified by a sonic horn (probe) and thereby disrupt the soil matrix. The residues are released from soil and dissolved in the solvent.

4.2.5 Supercritical fluid extraction (SFE)

SFE is used mainly for nonpolar compounds [e.g. polychlorinated biphenyls (PCBs)]. Typically, small aliquots of soil (0.5–10 g) are used for extraction. The extraction solvent is a supercritical fluid, most commonly carbon dioxide, which has properties of both a liquid and gas. The supercritical fluid easily penetrates the small pores of soil and dissolves a variety of nonpolar compounds. Supercritical carbon dioxide extracts compounds from environmental samples at elevated temperature (100–200 °C) and pressure (5000–10 000 psi). High-quality carbon dioxide is required to minimize

analytical interferences. Compounds with different chemical natures can be selectively extracted by varying the extraction pressure and temperature. The addition of an organic modifier, such as methanol, may improve the recoveries of polar compounds.

4.2.6 Accelerated solvent extraction (ASE)

This fully automated process developed by Dionex is used for a variety of compounds having a wide range of polarities.[49] Typically, a small aliquot of soil (0.5–20 g) is extracted using a variety of solvents. As with other techniques, the solvent choice depends upon the polarity of the compound to be extracted. The unit extracts soil at elevated temperatures (>60 °C) and pressures (>1000 psi). The increased temperature accelerates the extraction kinetics while the elevated pressure keeps the solvent(s) below the boiling point, thus allowing safe and rapid extraction. Both time and solvent consumption are dramatically reduced compared with mechanical shaking. There are now several published United States Environmental Protection Agency (USEPA) methods that use ASE (e.g., USEPA Method 600/4-81-055, 'Interim Methods for the Sampling and Analysis of Priority Pollutants in Sediment and Fish Tissue').

4.2.7 Microwave extraction

This is a relatively new technique that is used for PCBs and other nonpolar, volatile and semi-volatile organic compounds. Typically, a small aliquot of soil sample (0.5–20 g) is used for the extraction. Soil samples are extracted with one or more organic solvents using microwave energy at elevated temperature (100–115 °C) and pressure (50–175 psi). This method uses less solvent and takes significantly less time than Soxhlet extraction but is limited to thermally stable compounds.

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4.3 Sample cleanup

Trace analysis of soil samples often requires post-extraction cleanup to remove co-extracted matrix interferences. There are several difficulties that may arise during chromatographic analysis due to interferences present in sample extracts. To avoid these and other issues, one or more of the following cleanup techniques are often used.

4.3.1 Liquid–liquid partition

This technique provides a convenient method for separating an agrochemical compound from a highly aqueous extraction mixture. The partitioning solvent is usually a volatile, water-immiscible organic solvent that can be removed by evaporation after the desired component has been extracted. This technique is based on the principle that when a substance is soluble to some extent in two immiscible liquids, it can be transferred from one liquid to another by shaking. The degree of partitioning from one solvent to the other depends on the agrochemical's distribution coefficient between the immiscible liquids. This technique is particularly useful for the cleanup of ionizable compounds, since the pH of the aqueous solution can be adjusted to maximize partitioning into the organic or water phases, as desired.

4.3.2 Solid-phase extraction (SPE)

This technique is based on the same separation mechanisms as found in liquid chromatography (LC). In LC, the solubility and the functional group interaction of sample, sorbent, and solvent are optimized to effect separation. In SPE, these interactions are optimized to effect retention or elution. Polar stationary phases, such as silica gel, Florisil and alumina, retain compounds with polar functional group (e.g., phenols, humic acids, and amines). A nonpolar organic solvent (e.g. hexane, dichloromethane) is used to remove nonpolar inferences where the target analyte is a polar compound. Conversely, the same nonpolar solvent may be used to elute a nonpolar analyte, leaving polar inferences adsorbed on the column.

The most common technique used for agrochemicals is reversed-phase SPE. Here, the bonded stationary phase is silica gel derivatized with a long-chain hydrocarbon (e.g. C_4–C_{18}) or styrene–divinylbenzene copolymer. This technique operates in the 'reverse' of normal-phase chromatography since the mobile phase is polar in nature (e.g., water or aqueous buffers serve as one of the solvents), while the stationary phase has nonpolar properties.

Ion-exchange solid-phase extractions are used for ionic compounds. The pH of the extracts is adjusted to ionize the target analytes so that they are preferentially retained by the stationary bonded phase. Selection of the bonded phase depends on the pK_a or pK_b of the target analytes. Sample cleanup using ion exchange is highly selective and can separate polar ionic compounds that are difficult to extract by the liquid–liquid partition technique.

A variety of solid-phase cartridges are available from a number of different manufacturers (e.g. J.T. Baker, Varian). Most cartridges, however, use a similar extraction procedure that consists of these basic steps:

1. *Conditioning the column.* This step prepares the column to absorb the analytes and also pre-washes the column with the solvents that are used for the cleanup.
2. *Sample application.* The sample extract is dissolved in the weaker solvent and applied to the top of the column. The analytes of interest are extracted from the crude sample extract and are adsorbed on the column.
3. *Wash.* Solvents, weaker than the elution solvents, are used to remove interferences selectively.
4. *Elution.* The compound of interest is selectively eluted with a stronger solvent.

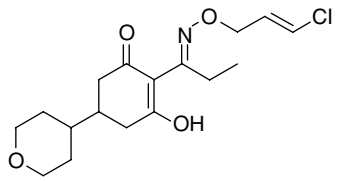
4.4 Derivatization techniques

A derivatization technique is commonly applied to an agrochemical with certain reactive functional groups (e.g., carboxylic acid, amine, phenol) to make the compound amenable to either gas chromatography (GC) or LC analysis. An in-depth discussion of derivatization reactions used in the analysis of agrochemicals is beyond the scope of this article. For more information on this topic, the reader is referred to Knapp.[50]

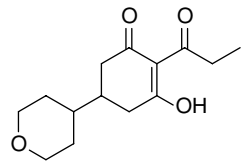
4.5 Analytical detection and quantitation techniques

The most common final separation techniques used for agrochemicals are GC and LC. A variety of detection methods are used for GC such as electron capture detection (ECD), nitrogen–phosphorus detection (NPD), flame photometric detection (FPD) and mass spectrometry (MS). For LC, typical detection methods are ultraviolet (UV) detection, fluorescence detection or, increasingly, different types of MS. The excellent selectivity and sensitivity of LC/MS/MS instruments results in simplified analytical methodology (e.g., less cleanup, smaller sample weight and smaller aliquots of the extract). As a result, this state-of-the-art technique is becoming the detection method of choice in many residue analytical laboratories.

An example of an LC/MS/MS method with an LOQ of 0.01 mg kg^{-1} is illustrated in Figure 9. This method was used to analyze tepraloxydim and its primary metabolite

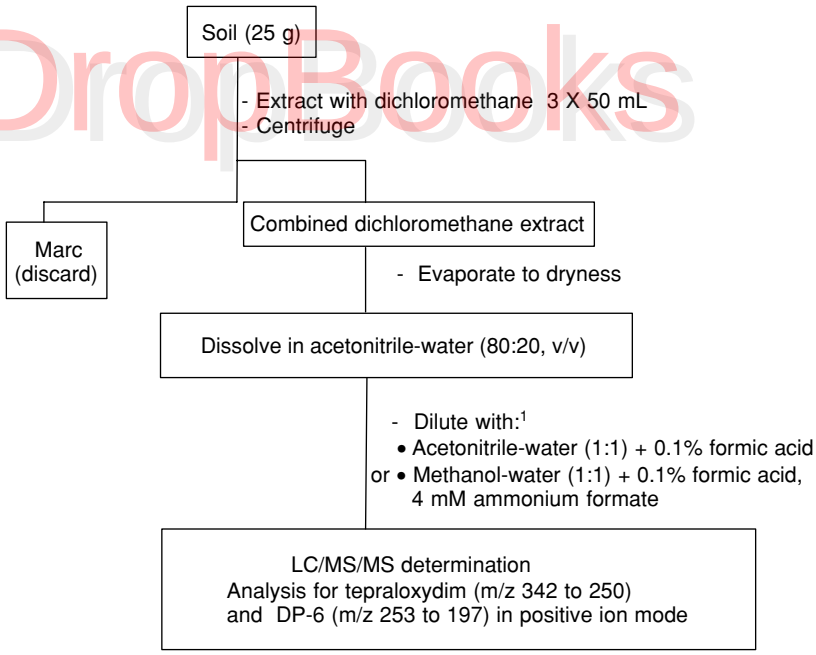


Tepraloxydim



DP-6

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Soil (25 g)
- Extract with dichloromethane 3 X 50 mL
- Centrifuge
Marc
(discard)
Combined dichloromethane extract
- Evaporate to dryness
Dissolve in acetonitrile-water (80:20, v/v)
- Dilute with:[1]
• Acetonitrile-water (1:1) + 0.1% formic acid
or • Methanol-water (1:1) + 0.1% formic acid,
4 mM ammonium formate
LC/MS/MS determination
Analysis for tepraloxydim (m/z 342 to 250)
and DP-6 (m/z 253 to 197) in positive ion mode

[1]Modifications were used for different soil types.

Figure 9 Method diagram for the determination of tepraloxydim and its degradate, DP-6, in soil (LOQ 0.01 mg kg^{-1})

Table 5 Recoveries of tepraloxyim and degradates from soil dissipation studies conducted in the USA and Canada

Compound fortified[a]	Recovery range (%)			Mean recovery (%)		
	North Dakota	Mississippi	California	North Dakota	Mississippi	California
(A) US sites						
Tepraloxydim	78–119	74–106	86–113	96 ± 10 ($n = 46$)	86 ± 7 ($n = 44$)	100 ± 9 ($n = 26$)
DP-6	69–116	71–102	77–102	93 ± 11 ($n = 46$)	92 ± 7 ($n = 44$)	89 ± 7 ($n = 26$)
Compound fortified[a]	Recovery range (%)			Mean recovery (%)		
	Manitoba	Saskatchewan	Alberta	Manitoba	Saskatchewan	Alberta
(B) Canadian sites						
Tepraloxydim	77–110	72–121	70–107	92 ± 9 ($n = 39$)	90 ± 9 ($n = 44$)	88 ± 8 ($n = 43$)
DP-6	71–116	74–119	72–118	90 ± 10 ($n = 39$)	94 ± 11 ($n = 44$)	94 ± 16 ($n = 43$)

[a] Fortification range for all three sites was 0.01–0.1 mg kg^{-1}.

DP-6 over 3000 soil samples collected from several terrestrial field dissipation studies. The sample procedural recoveries using this method, conducted concurrently with the treated samples during soil residue analysis, are summarized in Table 5. This method was proven to be short, rugged, sensitive, and suitable for measuring residues in soil and sediment at levels down to 0.01 mg kg^{-1}. The reproducibility of the methods also indicated acceptable method performance and, as a result, thousands of samples were analyzed using this methodology.

4.6 Freezer storage stability

Most agrochemicals remain stable in frozen soil for many months. However, it is important to verify this stability by conducting a freezer storage stability study. One type of study is conducted by fortifying known amounts of test substance and its major transformation products into control soil collected from a participating field site. Fortification normally occurs at two levels: replicate soil samples are fortified at the LOQ and at the highest expected residue concentration for each analyte of interest. The fortified soil samples are stored under the same conditions as the field samples and analyzed at different time periods that bracket the storage time of the actual field samples. The recoveries of the storage samples are compared with those obtained from day zero analyses to obtain the storage stability. In general, the method of analysis is the same as used for the soil residue analysis.

A second approach to determining freezer storage stability involves the reanalysis of incurred residues found in actual samples that are stored over time. Using this approach, soil from an actual field sample containing residues is periodically analyzed

during the course of the analysis phase of the study. A key advantage of this method is that the stability of actual field-derived residues is being determined. The main drawback is that this approach does not work for degradates that do not form in the field at concentrations at or above their LOQ values.

5 Phase IV: reporting of results

Once soil samples have been analyzed and it is certain that the corresponding results reflect the proper depths and time intervals, the selection of a method to calculate dissipation times may begin. Many equations and approaches have been used to help describe dissipation kinetics of organic compounds in soil. Selection of the equation or model is important, but it is equally important to be sure that the selected model is appropriate for the dataset that is being described. To determine if the selected model properly described the data, it is necessary to examine the statistical assumptions for valid regression analysis.

5.1 Goodness of fit testing

There are two statistical assumptions made regarding the valid application of mathematical models used to describe data.[51] The first assumption is that row and column effects are additive. The first assumption is met by the nature of the study design, since the regression is a series of X, Y pairs distributed through time. The second assumption is that residuals are independent, random variables, and that they are normally distributed about the mean. Based on the literature, the second assumption is typically ignored when researchers apply equations to describe data. Rather, the correlation coefficient (r) is typically used to determine goodness of fit. However, this approach is not valid for determining whether the function or model properly described the data.

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In Figure 10, two solutions (models) are shown for the same data set. The first solution is based on a linear fit (Hamaker equation) that provided a high correlation coefficient of 0.93. The second solution (Gustafson–Holden model) is based on a nonlinear solution that provided a high correlation coefficient of 0.98. However, based on an examination of the residuals from both equations, it is evident that the linear model failed to describe properly the data based on the second assumption for valid regression analysis (Figure 11). In other words, the residuals were not randomly distributed; initially they are greater than zero but become increasingly negative as time progresses. In contrast, the residuals from the nonlinear model are equally negative and positive throughout time and it appears, therefore, that the nonlinear model fulfills the second assumption for valid analysis (Figure 12). The second assumption for valid analysis becomes especially important when kinetics are implied based on the fit of the model. However, a kinetic model truly cannot be proven by a fit to data from a field dissipation study.[52–54] Therefore, the appropriateness of a model should be determined by its ability to empirically describe the data without implication of mechanism (order).

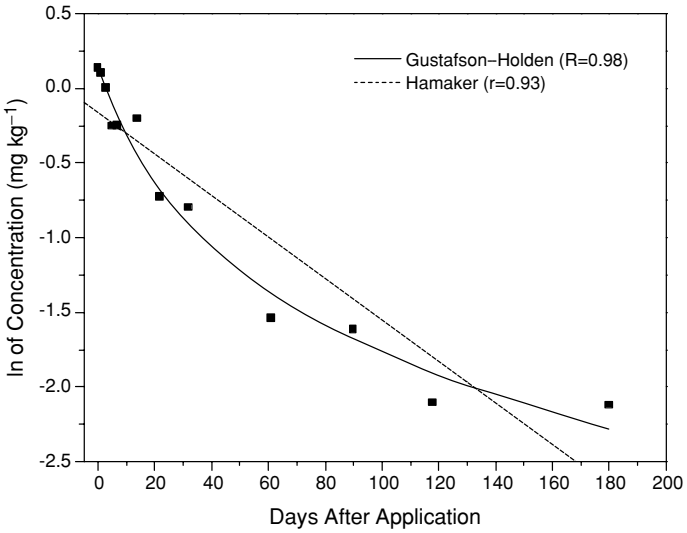


Figure 10 Comparison of linear (Hamaker) and nonlinear (Gustafson–Holden) solutions for a typical soil dissipation data set

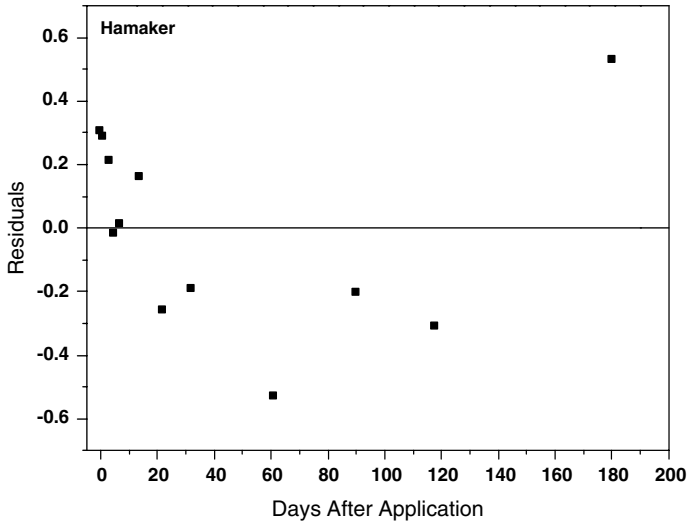


Figure 11 Residuals plot for the linear model

5.2 Models for agrochemical dissipation in soil

Since many equations and analysis procedures have been described in the literature, we present here just a few of the most commonly used equations. The solutions to these equations are obtained using a nonlinear curve fitting routine found in many commercially available statistical programs.

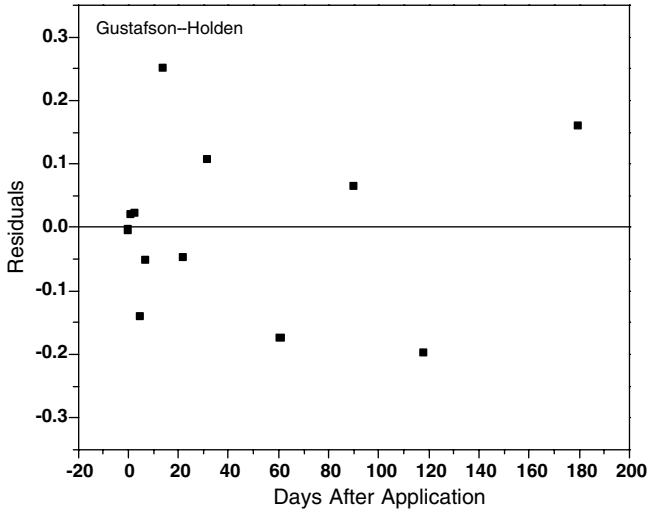


Figure 12 Residuals plot for the nonlinear model

5.2.1 Hamaker equation

The equation by Hamaker[55] is one of the most commonly used methods for describing dissipation kinetics using a linear fit. The basic computational form of the equation is

$$y = a \exp(-bX) \tag{6}$$

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This equation is satisfactory for data sets that are linear when ln of concentration is plotted vs time.

5.2.2 Hamaker equation (power rate form)

As mentioned previously, most agrochemicals do not exhibit linear degradation patterns. As a result, Hamaker[55] proposed another variation of the linear-fit equation that allows better description of nonlinear data sets:

$$y = a_0^{1-n} + (n-1)bX^{\frac{1}{1-n}} \tag{7}$$

where $n \neq 1$; n is the rate order and a and b are solved as unknowns 1 and 2. The disadvantage of this type of approach is that the user is simply choosing a power or 'order' that empirically describes the data better than the single exponential form of the equation.

5.2.3 Timme–Frehse–Laska equation

In a similar approach to Hamaker, Timme *et al.*[56] proposed six functions that are also empirically based. However, they took the additional step of suggesting that the choice of the equation should be based on the regression correlation coefficient (r).

However, regression coefficients cannot be used to determine the adequacy of a model choice, as discussed previously.

Order 1:

$$y = \left(\frac{\log X}{\log b}\right)^2 \tag{8}$$

Order 1.5:

$$y = \left[\frac{a}{b}(\sqrt{X} - 1)\right]^2 \tag{9}$$

Order 2:

$$y = \left[\frac{a}{b}(X - 1)\right]^2 \tag{10}$$

Similarly to the Hamaker parameters, a and b are solved as unknowns 1 and 2.

5.2.4 Gustafson–Holden equation

The Gustafson–Holden equation[57] is a unique approach that allows both linear and nonlinear datasets to be solved since it is based on a gamma distribution. The equation is first order and has three unknowns (a, b and c):

$$y = a - b\ \ln(1 + cX) \tag{11}$$

This equation requires more data points than the previous equations.

5.2.5 Wolt equation

The Wolt equation[52] is also a unique approach that is described as being a quasi-first-order equation. This equation also has three unknowns that are solved (a, b, and c):

$$y = a + b\exp(-cX) + e \tag{12}$$

The variable e has been described as an error term, but is not used in most applications of the equation.

5.3 DT_{50} versus $T_{1/2}$ values

It is important that a clear distinction be made between DT_{50} and $T_{1/2}$ values. A DT_{50} implies that the value describes the time required for 50% of the starting concentration to dissipate or degrade. A $T_{1/2}$ result implies that the number is derived from a rate constant, which may or may not describe where 50% of the starting concentration has dissipated or degraded. If a logarithm concentration data set is nonlinear with time,

a rate constant will not accurately describe the data. If the dataset is linear, the rate constant and the DT_{50} result should be about equal. A rate constant solution describes a data set with the assumption that an equal change in concentration occurs with an equal change in time. The Hamaker equation is an example of one of the most widely used rate constant equations.

5.4 Determining water balance and leaching potential

One of the objectives for a field dissipation study is to determine how the leaching behavior of an agrochemical is correlated with water inputs occurring at the field site. In order to answer this question, researchers often overlay water additions on top of graphs displaying residue movement. However, this method often falls short of answering the basic question of whether sufficient water was applied to allow leaching to occur. For example, clay loam soils have on average a 6.4-cm water holding capacity per 30-cm depth. If the water content of the clay soil is approximately at permanent wilt point and a 4-cm irrigation event occurs, the 30-cm depth of soil will not reach field capacity. If the field capacity is never exceeded, no movement of soil solute from the 0- to 30-cm depth would be expected to occur. (These techniques do not address preferential or by-pass flow processes where agrochemicals are transported to subsoils via water following root channels, cracks, etc. Techniques to address preferential flow are not well established at this time.) If three days later an additional 3.2-cm rainfall event occurred, the 0- to 30-cm depth of soil would still not have been brought back to field capacity (assuming 0.7-cm evaporation on the previous two days).

For these reasons, it is desirable to perform a series of simple calculations to determine if the field capacity for a given depth of soil is ever exceeded, rather than simply overlaying water inputs over plots of residue data. The following series of calculations addresses the primary issue of whether sufficient water was applied to the test system at appropriate intervals to create leaching opportunities:[58]

Surface-layer calculation:

$$\Delta\theta_{1i}^{t+1} = \sum_{1i}^{t+1}[(P + SM + I) - (Q - ET_c)] \tag{13}$$

Sub-surface-layer calculation:

$$\Delta\theta_{1i}^{t+1} = \sum_{1i}^{t+1}(Inf - RF_c) \qquad (ET_c \text{ if } \theta \text{ in an overlying layer} = 0) \tag{14}$$

where

t = time in days
θ = volumetric water content
P = precipitation
SM = snow melt (when snow pack exists and ambient temperature is $>0\,^{\circ}C$)

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I = irrigation
Q = runoff
ET_c = evapotranspiration corrected for the crop ($ET_c = ET_0 \times K_c$) or E_{soil} ($E_{soil} = ET_0 \times k$)
Inf = infiltration
RF_c = root extraction factor $RF = RF \times c$, $c = 1.0$

Once performed, these calculation results can be graphed as shown in Figure 13. This type of information provides more insight into the soil water status at a site than simply graphing rainfall. This figure also helps determine if soil water movement occurred out of a given depth of soil. Moreover, it is useful to overlay Figure 13 with a graph of compound movement by depth to determine if the predicted water flux at a given depth corresponds to actual residue movement.

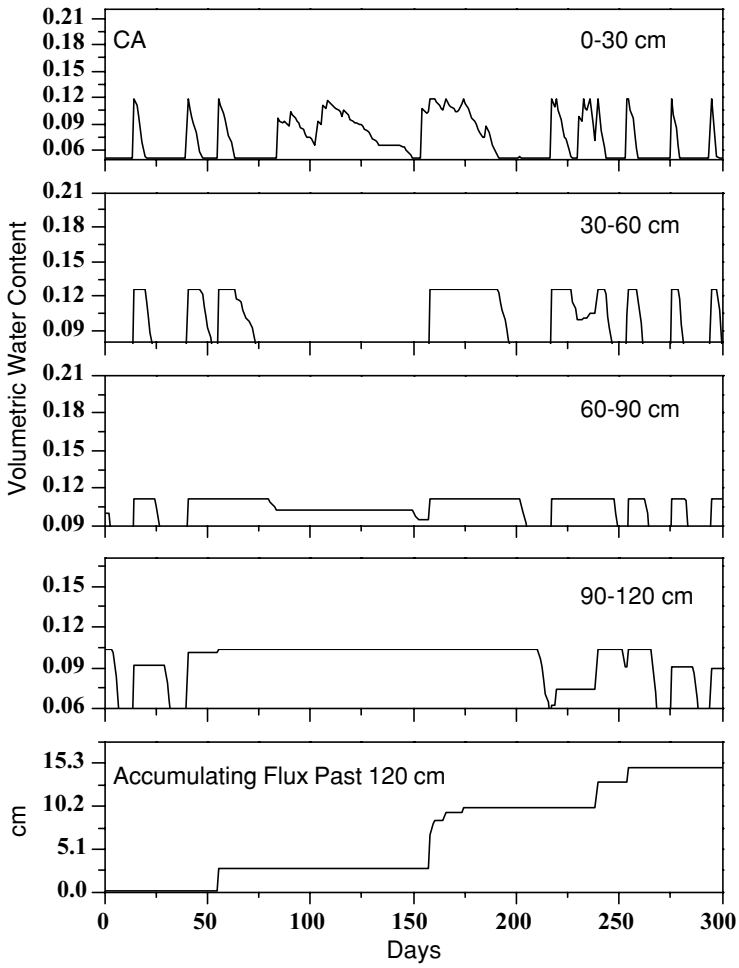
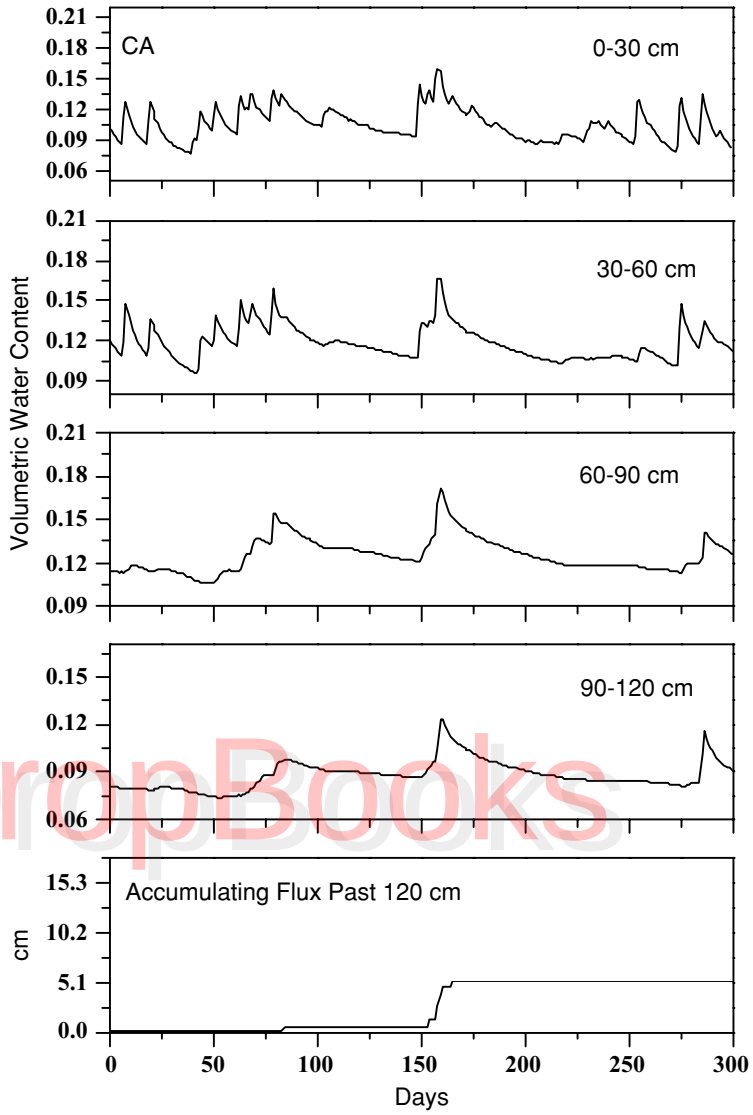


Figure 13 Volumetric water contents for Haw series soil calculated using the Penman equation



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Figure 14 Comparison of actual volumetric water contents (measured by time domain reflectometry) and calculated soil-water flux values (Penman equation) at four soil depths

More sophisticated methods that actually measure volumetric water content can also be used, such as time domain reflectometry (TDR). In Figure 14, an example of TDR results is presented. Both the calculated and measured (i.e., TDR) volumetric water contents provide a similar picture of the profile water status by depth with time. Proper soil characterization data, such as those shown in Table 6, are necessary for these calculations and improve understanding of the test system. The determination of water-holding capacity (WHC) at 0.03 MPa field capacity (FC) and 1.5 MPa

Table 6 Soil characterization results used in water balance calculations and data interpretations

	Depth increment (cm)							
Soil characteristic	0–15	15–30	30–45	45–60	60–75	75–90	90–105	105–120
Sand (%)	85	85	85	83	79	85	85	83
Silt (%)	9	9	9	13	15	9	9	11
Clay (%)	6	6	6	4	6	6	6	6
Organic matter (%)	2.1	0.9	0.3	0.2	0.2	0.1	0.1	0.1
Bulk density ($g\,cm^{-3}$)	1.33	1.41	1.49	1.46	1.45	1.43	1.45	1.47
pH	6.1	6.1	7.2	6.1	5.9	6.2	6.4	6.2
WHC[a] at 0.33 bar (%)	9.9	6.4	5.4	4.7	5.8	5.8	5.1	5.4
WHC at 15 bar (%)	5.1	3.4	2.4	2.0	2.1	2.7	2.1	2.3
CEC[b] (mequiv. per 100 g soil)	8.1	5.8	6.4	3.5	2.9	4.0	2.9	3.1
Textural classification	Loamy sand	Loamy sand	Loamy sand	Loamy sand	Loamy sand	Loamy sand	Loamy sand	Loamy sand

[a] WHC = water-holding capacity.
[b] CEC = cation-exchange capacity.

permanent wilt point (PWP) is important for any type of soil-water calculations or for field sensor measurements.

In Table 7, a comparison of actual measurements, and also two well-known pedo-transfer functions, can be found by depth. It is important to note that there is a large difference in water content between the disturbed soil core samples and the undisturbed samples. Additionally, the two pedo-transfer functions also exhibit a large difference in predicted water content. Therefore, when doing calculations or trying

Table 7 Measured and estimated volumetric water contents as a function of depth and matrix potential for a Haw series soil (Payette Country, Idaho)

	Volumetric water content							
	Intact soil core: measured	Disturbed soil core: measured	Pedo-transfer function I: estimated[a]	Pedo-transfer function II: estimated[b]	Intact soil core: measured	Disturbed soil core: measured	Pedo-transfer function I: estimated	Pedo-transfer function II: estimated
Depth (cm)	Soil matrix potential, 0.03 MPa (field capacity)				Soil matrix potential, 1.5 MPa (permanent wilting point)			
0–15	28.60	33.50	36.93	27.18	18.73	23.40	19.14	12.05
15–30	27.85	34.90	31.30	27.18	19.07	26.10	17.72	12.05
30–45	35.88	41.10	27.82	27.18	25.17	26.60	14.89	12.04
45–60	43.68	43.80	20.98	26.24	32.27	28.50	7.59	11.48
60–75	37.55	41.10	21.12	26.71	30.23	25.80	7.62	11.75
75–90	39.83	42.40	20.58	24.84	28.30	23.50	8.14	10.68
90–105	38.23	40.70	24.04	24.84	26.03	23.50	13.12	10.71
105–120	38.10	37.10	27.40	24.84	26.30	20.60	16.70	10.73

[a] Estimated using the method of Rawls *et al.*[59]
[b] Estimated using the method of Bauer and Black.[60]

to calibrate field sensors, the magnitude of the differences observed in Table 7 must be considered and a compromise should be struck between precision and accuracy.

5.5 Weather data requirements for water balance and mobility assessments

If basic calculations such as those presented are to be conducted, it is important to collect enough weather parameters to calculate reference evapotranspiration (ET_0). An on-site weather station should be considered a basic requirement: minimum sensor requirements to calculate a Penman equation would include solar radiation, wind speed, relative humidity or actual vapor pressure, and air temperature. An on-site rain gauge is essential but it is also a good idea to have a rain gauge on the weather station even if it is not directly on-site. The most accurate variations of the Penman equation calculate ET_0 on an hourly basis. However, Penman routines using daily summaries are typically satisfactory for the purpose of calculating soil-water recharge.

6 Summary and conclusions

The proper conduct of a field soil dissipation study represents a significant commitment of labor, money, and time. As such, there are many important study details that cannot be left to chance, or addressed as an afterthought, once the study is underway. Each of the four main phases of study conduct, (1) planning and design, (2) field conduct, (3) sample processing and analysis, and (4) data handling and reporting, is vitally linked to the next. Each phase is critical to study success. This article addresses key aspects of study design and conduct necessary for successful study completion. When properly planned and conducted, these studies provide valuable information regarding the environmental persistence and mobility of agrochemicals in field soils.

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7 Abbreviations

ASE	Accelerated solvent extraction
AV	Application verification
ECD	Electron capture detection
GC	Gas chromatography
LC	Liquid chromatography
LOQ	Limit of quantitation
MS	Mass spectrometry
NPD	Nitrogen–phosphorus detection
SFE	Supercritical fluid extraction
SPE	Solid-phase extraction
TDR	Time domain reflectometry
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
K_D, K_{OC}	Soil sorption coefficients

pK_a	Acid dissociation constant
r	Regression correlation coefficient
R^2	Regression coefficient of determination
S_w	Water solubility

References

1. S.K. Singles, R.F. Dietrich, and R.D. McFetridge, 'Degradation of pyrithiobac sodium in soil in the laboratory and field,' in "Pesticide Environmental Fate, Bridging the Gap Between Laboratory and Field Studies," ed. W. Phelps, K. Winton, and W.R. Effland, ACS Symposium Series No. 813, American Chemical Society, Washington, DC, Chapter 15, pp. 207–221 (2002).
2. B. Stenberg, M. Johansson, M. Pell, K. Sjödahl-Svensson, J. Stenström, and L. Torstensson, *Soil Biol. Biochem.,* **30**, 393 (1998).
3. S.L. Trabue, T.M. Crowe, and J.H. Massey, 'Changes in soil biomass and microbial community structure as affected by storage temperature and duration: effect on the degradation of metsulfuron methyl' in 'Pesticide Environmental Fate: Bridging the Gap Between Laboratory and Field Studies', ed. W. Phelps, K. Winton, and W.R. Effland, ACS Symposium Series 813, American Chemical Society, Washington, DC (2002).
4. L.O. Osa-Afiana and M. Alexander, *Soil Sci. Soc. Am. J.,* **46**, 285 (1982).
5. J.E. Woodrow, J.S. Seiber, and L.W. Baker, *Environ. Sci. Technol.,* **31**, 523 (1997).
6. I.J. van Wesenbeeck, J.M. Zabik, J.D. Wolt, G.A. Bormett, and D.W. Roberts, *J. Agric. Food Chem.,* **45**, 3299 (1997).
7. J.M. Zabik, I.J. van Wesenbeeck, A.L. Peacock, L.M. Kennard, and D.W. Roberts, *J. Agric. Food Chem.,* **49**, 3284 (2001).
8. H.J. Strek, *Pestic. Sci.,* **53**, 52 (1998).
9. R.C. Knox, D.A. Sabatini, and L.W. Canter, 'Subsurface Transport and Fate Processes,' Lewis Publishers, Boca Raton, FL, Chapter 6, pp. 268–272 (1993).
10. P.H. Nicholls and A.A. Evans, *Pestic. Sci.,* **33**, 319 (1991).
11. S.Z. Cohen, S.M. Creeger, R.F. Carsel, and C.G. Enfield, 'Potential for pesticide contamination of ground water from agricultural uses,' in "Treatment and Disposal of Pesticide Wastes," ed. R.F. Krueger and J.N. Seiber, ACS Symposium Series No. 259, American Chemical Society, Washington, DC, Chapter 18, pp. 318–319 (1984).
12. H.H. Cheng, 'Pesticides in the Soil Environment: Processes, Impacts and Modeling,' Soil Science Society of America, Madison, WI (1990).
13. M.A. Locke and C.T. Bryson, *Weed Sci.,* **45**, 307 (1997).
14. A.W. Taylor, H.P. Freeman, and W.M. Edwards, *J. Agric. Food Chem.,* **19**, 832 (1971).
15. J. Rouchaud, O. Neus, K. Cools, and R. Bulcke, *J. Agric. Food Chem.,* **47**, 3872 (1999).
16. X. Jianming, W.C. Koskinen and H.H. Cheng, *Pedosphere,* **10**, 289 (2000).
17. D. Hillel, 'Environmental Soil Physics,' Academic Press, New York, Chapters 18 and 19, pp. 507–587 (1998).
18. A.S. Felsot, R.G. Evans, and J.R. Ruppert, 'Field studies of imidacloprid distribution following application to soil through a drip irrigation system', in "Terrestrial Field Dissipation Studies: Design, Interpretation and Purpose," ed. E.L. Arthur, V.E. Clay, and A. Barefoot, ACS Symposium Series No. 842, American Chemical Society, Washington, DC (2003).
19. W.R. Effland, N.C. Thurman, R. Gangaraju, I. Nicholson, and D. Kroetsch, 'GIS decision support system to evaluate U.S. and Canada field study areas for agrochemicals,' in "Pesticide Environmental Fate, Bridging the Gap Between Laboratory and Field Studies," ed. W. Phelps, K. Winton, and W. R. Effland, ACS Symposium Series No. 813, American Chemical Society, Washington, DC, Chapter 2, pp. 2–21 (2002).
20. R.K. Trubey, R.A. Bethem, and B. Peterson, *J. Agric. Food Chem.,* **46**, 2360 (1998).
21. D.L. Suett, *Pestic. Sci.,* **6**, 385 (1975).
22. L.E. Bode and M.R. Gebhardt, *Weed Sci.,* **17**, 551 (1969).

23. R.D. Wauchope, J.M. Chandler, and K.E. Savage, *Weed Sci.,* **25**, 193 (1977).
24. C.G. McWhorter and O.B. Wooten, *Weeds,* **9**, 42 (1961).
25. C.L. Stiles, C.E. Sams, D.K. Robinson, D.L. Coffey, and T.C. Mueller, *J. Agric. Food Chem.,* **48**, 4681 (2000).
26. FIFRA Scientific Advisory Panel Meeting Report, 'Review of Proposed Revised Guidelines for Conducting Terrestrial Field Dissipation Studies', SAP Report No. 90-01, United States Environmental Protection Agency, Washington, DC, p. 32 (1998).
27. 'Pesticide Rejection Rate Analysis – Environmental Fate,' EPA 738-R-93-010, United States Environmental Protection Agency, Washington, DC (1993).
28. M. Alexander, 'Biodegradation and Bioremediation,' second edition, Academic Press, San Diego, Chapter 6, pp. 99–100 (1999).
29. J.H. Massey and J.S. LeNoir, 'Sources and magnitudes of variability in terrestrial field dissipation of agrochemicals,' in "Terrestrial Field Dissipation Studies: Design, Interpretation and Purpose," ed. E.L. Arthur, V.E. Clay, and A. Barefoot, ACS Symposium Series No. 842, American Chemical Society, Washington, DC (2003).
30. A. Walker and P.A. Brown, *Crop Protect.,* **2**, 17 (1983).
31. P.S.C. Rao and R.J. Wagenet, *Weed Sci.,* **33** (Suppl. 2), 18 (1985).
32. L.S. Wood, H.D. Scott, D.B. Marx, and T.L. Lavy, *J. Environ. Qual.,* **16**, 251 (1987).
33. J.M. Novak, T.B. Mooreman, and C.A. Cambardella, *J. Environ. Qual.,* **26**, 1271 (1997).
34. C. Vischetti, M. Businelli, M. Marini, E. Capri, M. Travisan, A.A.M. Del Re, L. Donnarumma, E. Conte, and G. Imbroglini, *Pestic. Sci.,* **50**,175 (1997).
35. K.A. Gomez and A.A. Gomez, 'Statistical Procedures for Agricultural Research,' second edition Wiley, New York, Chapter 2, pp. 7–83 (1984).
36. M.G. Cline, *Soil Sci.,* **58**, 275 (1944).
37. R.G. Petersen and L.D. Calvin, 'Sampling,' in "Methods of Soil Analysis, Part I. Physical and Mineralogical Methods," Agronomy Monograph No. 9, second edition, American Society for Agronomy, Madison, WI, Chapter 2, pp. 33–51 (1986).
38. D.C. Montgomery, 'Design and Analysis of Experiments,' second edition, Wiley, New York, Chaper 3, p. 51 (1984).
39. S.A. Clay, D.E. Clay, W.C. Koskinen, and G.L. Malzer, *J. Environ. Sci. Health,* **B27**, 125 (1992).
40. D.E. Clay, C.G. Carlson, K. Brix-Davis, J. Oolman, and B. Berg, *J. Prod. Agric.,* **10**, 446 (1997).
41. W.P. Anderson, 'Weed Science Principles', second edition, West Publishing, St. Paul, MN, Chapter 8, pp. 341–364 (1983).
42. TeeJet, 'Agricultural Spray Products, Catalog 47,' Spraying Systems, Wheaton, IL, p. 136 (1998).
43. M.P. Braverman,, J.S. Corley, D.C. Thompson, M. Arsenovic, V.R. Starner, K.S. Samoil, F.P. Salzman, D.L. Kunkel, and J.J. Baron. *Proc. Weed Sci. Soc. Am.*, **41**, 17 (2001).
44. A. Nordby and R. Skuterud, *Weed Res*., **14**, 385 (1975).
45. J.H. Massey and S.K. Singles, 'Photostability of two fungicides on spray application monitors,' in "Terrestrial Field Dissipation Studies: Design, Interpretation and Purpose," ed. E.L. Arthur, V.E. Clay, and A. Barefoot, ACS Symposium Series No. 842, American Chemical Society, Washington, DC (2003).
46. D.G. Graham, V. Clay, S.H. Jackson, and R. Jones, 'Field dissipation studies – The measurement of zero-time residues,' in "Terrestrial Field Dissipation Studies: Design, Interpretation and Purpose," ed. E.L. Arthur, V.E. Clay, and A. Barefoot, ACS Symposium Series No. 842, American Chemical Society, Washington, DC (2003).
47. T.H. Dao, T.L. Lavy, and J. Dragun, *Residue Rev.*, **87**, 92 (1983).
48. B.J. Weinhold and T.J. Gish, *Weed Sci.*, **39**, 423 (1991).
49. B. Richter, J. Ezzell, and D. Felix, 'Single Laboratory Method Validation Report: Extraction of Organo-phosphorus Agrochemicals, Chlorinated Herbicides and Polychlorinated Biphenyls Using Accelerated Solvent Extraction (ASE) with Analytical Validation by GC/NPD and GC/ECD,' Document 101124, Dionex Sunnyvale, CA (1994).
50. D.R. Knapp, 'Handbook of Derivatization Reactions,' Wiley, New York (1979).

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51. G.W. Snedecor and W.G. Cochran, 'Statistical Methods,' Iowa State University Press, Ames, IA (1987).
52. J.D. Wolt, 'Soil Solution Chemistry,' Wiley, New York (1994).
53. J.D. Wolt, H.P. Nelson, Jr, C.B. Cleveland, and I.J. van Wesenbeeck, *Rev. Environ. Contam. Toxicol.*, **169**, 123 (2001).
54. D.L. Sparks, 'Kinetics of Soil Chemical Processes,' Academic Press, San Diego (1989).
55. J.W. Hamaker, 'Decomposition: quantitative aspects,' in "Organic Chemicals in the Soil Environment", ed. C.A.I. Goring and J.W. Hamaker, Marcel Dekker, New York, pp. 253–340 (1972).
56. G.H. Timme, H. Freshe, and V. Laska, *Pflanzenschutz-Nachr. Bayer,* **39**, 187 (1986).
57. D.I. Gustafson and L. Holden, *Environ. Sci. Technol*., **24**, 1032 (1990).
58. R.G. Allen, L.S. Pereira, D. Raes, and M. Smith, 'Crop Evapotranspiration – Guidelines for Computing Crop Water Requirements,' FAO Irrigation and Drainage Paper 56, Food and Agriculture Organization of the United Nations, Rome (1998). Also available on the World Wide Web: **http://www.fao.org/docrep/x0490e/x0490e00.htm**.
59. W.J. Rawls, D.L. Brakensiek, and K.E. Saxton, *Trans. ASAE*, **81**, 2510 (1982).
60. A. Bauer and A.L. Black. *Soil Sci. Soc. Am. J.,* **56**, 248 (1992).

Sampling sediment and water in rice paddy fields and adjacent water bodies

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1 Introduction

1.1 Rice production in paddy fields

Rice is one of the most important and basic staple foods for about half of the world's population and provides over 20% of the global calorie intake. World rice production is projected to expand by 1.4% per year to 424 million tonnes by 2005, according to the Food and Agricultural Organization (FAO).

In key rice-producing regions in Asia, rice production is performed mostly in paddy fields where crop production has been highly sustainable owing to:

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1. avoidance of soil-borne disease so that the production can be repeated every year on the same field, in some cases for more than 1000 years
2. avoidance of soil erosion, enabling fertile surface soil to be conserved
3. substantial additional benefits in flood control and groundwater conservation.

The above-mentioned features are consistent with the wider global efforts at sustainable agriculture.

With the predicted increase in world population, the production of rice in paddy fields will increase further in importance in producing and maintaining the necessary food supply. Therefore, the importance of plant protection in rice paddies by the use of suitable agrochemicals must be taken into consideration. The potential impact of agrochemicals on the rice paddy environment and adjacent areas presents challenges to agriculture and the regulation of agrochemicals.

The application of pesticides to paddy fields represents a unique set of issues compared with many other use patterns. Agrochemicals used in rice production are introduced directly or indirectly into paddy water, and there are more opportunities for

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paddy water to be released into aquatic bodies in the environment through agricultural drains.

Under typical agricultural conditions after an agrochemical application, paddy water is held in the paddy for a period of 5–14 days before release. The length of the water-holding period depends on the type of chemical used and local cultural practices. If the residues of agrochemical are released with paddy water into adjacent water bodies, there would be a potential risk to both aquatic organisms and the quality of drinking water that need to be assessed. Understanding of this transport pathway is of critical importance ecotoxicologically for rice paddy agrochemicals. This involves consideration not only of relevant species living in the water phase but also those living species that spend a major portion of their lifecycle living in and on aquatic sediments. Direct transfer of chemicals from sediments to organisms is now considered to be a major route of exposure for many species.

River water is the main source of drinking water in Japan, in contrast to Europe and the USA where groundwater is the main source. In Japan, river water contamination by agrochemicals is an important component in assessing consumer safety via the consumption of drinking water. Government agencies (Ministry of the Environment, Ministry of Agriculture, Forestry and Fisheries, and Ministry of Health, Labor and Welfare) and many chemical companies have carried out water quality monitoring of major rivers in Japan to determine the significance of agrochemicals in sources of drinking water.

Water management of paddy fields is very important not only with respect to the cultivation practice of rice but also for the prevention of the contamination of water. Irrigation control after the application of agrochemicals could be the most important approach to avoiding environmental impact. Agrochemicals applied to rice paddy fields could be easily transported to adjacent water bodies compared with those in use on upland areas. Japanese researchers have clarified the relationship between the rate of flow out of paddies and the water solubility of agrochemicals.[1,2] Pesticides that have high water solubility have the greatest potential to flow out into adjacent water bodies.

In this article, sampling methods for sediments of both paddy field and adjacent water bodies, and also for water from paddy surface and drainage sources, streams, and other bodies, are described. Proper sample processing, residue analysis, and mathematical models of dissipation patterns are also overviewed.

1.2 Regulatory requirements and guidelines

Japan has unique regulations regarding environmental fate in the registration of agrochemicals applied in paddy fields. In addition to ordinary environmental fate studies such as biodegradability in soil and hydrolysis and photolysis in water, additional lysimeter studies are required for the registration. Concentrations of agrochemicals in surface water, sediment, and leaching water after the application should be determined during a certain period with more than two model paddies using lysimeters (Figure 1) filled with different types of paddy soil generally distributed in Japan.

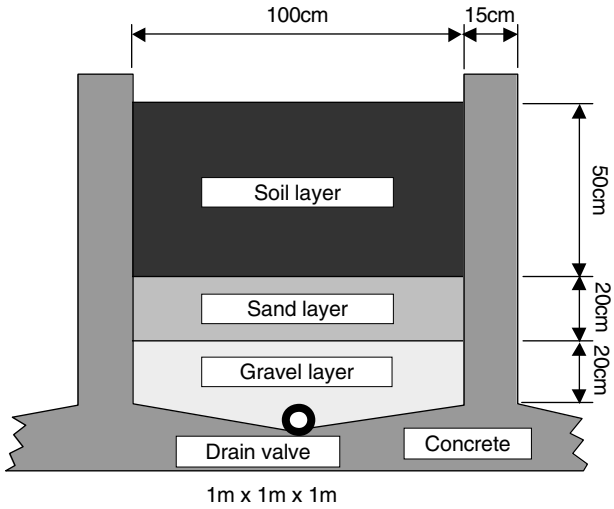


Figure 1 Structure of a lysimeter for a model paddy study

Drinking water quality should be taken into account from a human toxicological viewpoint because the main source of drinking water is river water. Japanese regulatory procedures allocate 10% of the acceptable daily intake (ADI) in principle to the intake from drinking water.

Monitoring studies in the actual fields are not required because Japan has no provisional registration system. Most companies, however, have been voluntarily monitoring their chemicals in river water after distribution.

Ecotoxicological data based on Organization for Economic Cooperation and Development (OECD) guidelines are also required, and the endpoints for aquatic organisms, such as fish, daphnia, algae and aquatic plants, are needed for utilization as part of the risk assessment process.

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2 Study design

2.1 Study objectives

Generally, pesticides applied in rice paddies disperse in sediment and water in the paddy field, and are released into adjacent water bodies through the agricultural drain. The objective of sediment and water sampling is to obtain reliable information about the behavior of pesticides and describe dissipation in the environment. Also, water quality monitoring has great importance for drinking water safety, especially in countries where river water is used as drinking water. The sample collected to obtain information may or may not be representative of the environment. The reliability of data acquired from samples depends on how the sample is selected and collected.

2.2 Preparation of study protocol

2.2.1 Test substances

(1) Use pattern information. Information regarding use patterns listed below is essential for the analysis of data:

- active ingredient
- formulation
- method of application (e.g., paddy water surface, foliar, nursery box)
- application rate [including active ingredient(s) contents]
- date of application
- target crop
- history of agrochemical and fertilizer use

(2) Physico-chemical properties. Chemical and biochemical degradation pathways and physical mechanisms of removal or disappearance by transport process govern the fate of agrochemicals in the environment. Therefore, the physico-chemical properties of the chemical listed below regarding persistence in sediment or water are important:

- soil adsorption coefficient (K_{oc})
- water solubility
- octanol–water partition coefficient (K_{ow})
- chemical stability, e.g., photolysis, hydrolysis
- aerobic and anaerobic biodegradability

2.2.2 Selection of test sites

Field studies in at least two paddies where the sediment has different characteristics of pH, texture and organic carbon contents are required for registration purposes. Since especially clay content and organic carbon content affect the agrochemical behavior in sediments, it is desirable that both systems have widely different characteristics with respect to these two criteria. These paddies should have cultivation history records on type of crop, variety, and agrochemical applications for at least 5 years.

When aimed at a single paddy field, the waterway connected with a paddy field serves as a target for a survey. Since a water intake and a drain are installed in each paddy, a major flow of water prevails from the intake to the drain. Hence, starting and ending points should be considered when water sampling is done. For the sampling of water, at least one point in the upstream of the inflow and more than one point in the downstream of the drain should be set.

When aimed at a group of paddy fields (an area or a region), sampling points should be set at locations where all the irrigation canals come into the area and all the drainage canals go out from the area.

For water sampling from drainage or streams, points near a drain port of a paddy field or an industrial factory, etc., should be avoided in order that water monitoring reflects the concentration of agrochemicals in the entire area rather than a point source.

2.2.3 Sampling

(1) Sampling design. To investigate the behavior of agrochemicals in a paddy field or a group of paddy fields, paddy water and surface sediments of the paddy field and water and sediments of waterways must be collected. In addition, a sediment column is collected and analyzed independently in depth segments to investigate the vertical movement of agrochemicals.

All soils and sediments are naturally variable. Their properties change horizontally across the landscape and vertically down the sediment profile. Heterogeneity may occur even in paddy fields where the transplanting of rice seedlings is practiced, although the horizontal variability in the surface layer is usually less than in upland arable lands or paddy fields receiving direct sowing, since the paddling practice in the field reduces the variability to some extent.

The times of sample collection for a single paddy survey are set with respect to the application time, such as just before an application, immediately after, and 1, 3, and 7 days post-application, and at longer intervals as appropriate. For an area survey of water and waterway sediments, samples are usually collected periodically over a cultivation season focusing on the application time.

The dissipation pattern observed depends mainly on the chemical itself, physico-chemical properties of sediment and formulation of the agrochemical because adsorption–desorption processes within the sediment are affected by these factors. The physico-chemical properties of the sediment, especially clay contents and organic carbon contents, affect the partitioning of the chemical to sediment. Degradation by both photolysis and hydrolysis may occur in the water phase. Microbial degradation may occur in both the water and sediment phases. At the initial stage of the dissipation when the concentration of a chemical is high immediately after an application, the distribution of the chemical affects the dissipation pattern. As a matter of course, the physico-chemical properties of the chemical such as adsorption coefficients (K_{oc}), water solubility, and octanol–water partition coefficients (K_{ow}) influence the dissipation pattern for the same reason. The formulation of the chemical may affect the dissipation pattern. For example, a dissipation rate of a granule that takes a certain time to dissolve is lower than that of a wettable powder, emulsifiable concentrate, or aqueous solution concentrate.

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(2) Critical information required at each sampling. The following information must be recorded for an environmental fate study in a paddy field:

- study identification
- Study Director/field investigator
- testing dates
- test location (address-lot number, latitude–longitude)
- description of the test plot
  —soil name
  —soil map unit name
  —irrigation and drainage system
  —landscape as useful accessory information
  —landform
  —climate (annual rainfall and temperature)

- basic sampling data
  —paddy water depth
  —horizon or depth sampled
  —sampling date
  —name of sampler
  —sampling method (e.g., probe, auger, core)
  —sampling plan (e.g., random, purposeful)
- crop (e.g., rice plant, rush)

The position relationship between irrigation canals and drainage canals connected with the paddy(s) and an adjacent river should be described distinctly.

2.2.4 Sediment characterization

The physico-chemical characteristics of the sediment sample significantly influence the fate of agrochemicals in a paddy field and a waterway system. Therefore, the factors that influence adsorption, retention, and degradation of agrochemicals are very important. As a minimum the characteristics of the sediment sample listed below should be described:

- pH
- texture (clay, silt, and sand proportions) in the United States Department of Agriculture (USDA) classification
- total and organic carbon contents
- total nitrogen contents

The data listed below are valuable for a more detailed analysis:

- cation-exchange capacity (CEC)
- phosphate absorption coefficient (PAC)
- microbial biomass
- clay mineralogy

2.2.5 Water quality determination

It is desirable to determine the chemical properties of irrigation water, paddy water in the field, and adjacent streams and rivers. Since especially the pH of the paddy water fluctuates diurnally (high in daytime and low at night), this may affect the water solubility of certain chemicals, e.g., sulfonylureas, which have dissociation constants (pK_a) in an environmentally relevant range.

2.2.6 Minimum weather data

Data on weather conditions, especially temperature and rainfall (temporal distribution and intensity) in the study area are essential for the evaluation of the dissipation data. It is very important to understand the water balance in the paddy field as accurately as possible when calculating the rate of outflow. Records of changes in water temperature and sediment temperature are also helpful for modeling the behavior of a chemical in the rice paddy field.

2.2.7 Irrigation program

Water management practices and the related information listed below should be described:

- date of plowing
- date of paddling (paddy preparation)
- date of sowing or transplanting
- water management
  —daily depth of paddy water after the chemical application
  —flooding period
  —water holding times
  —flow rate into and out of the paddy
  —date of drainage

The irrigation method in the period of flooding, e.g., 'days of holding water in paddy', could be important information for data interpretation.

Water management during the study should be conducted in accordance with the usual local best agricultural practice of rice cultivation except where specific investigation of a parameter requires an alternative. Usually, water is introduced to plowed paddy fields before paddling (*Sirokaki* in Japanese) for transplanting. Chemicals may be applied before or after transplanting and usually water is retained in the paddy field for 3–7 days after the application to ensure exposure of target organisms to the crop protection product. The loss of water through percolation and evapotranspiration is made up by adding water to the field from irrigation canals or wells. Thereafter, water is allowed to flow into and out of the rice field and the water level is kept at about a depth of 5 cm. After 30–50 days of flooded conditions, the rice field is drained. After about 2 weeks in a drained condition (midseason drainage, *Nakabosi*), water is reintroduced and maintained at a depth of 5–8 cm for several weeks. Intermittent irrigation and/or surface drainage follow after the deep flooding for the maturing stage (Figure 2).

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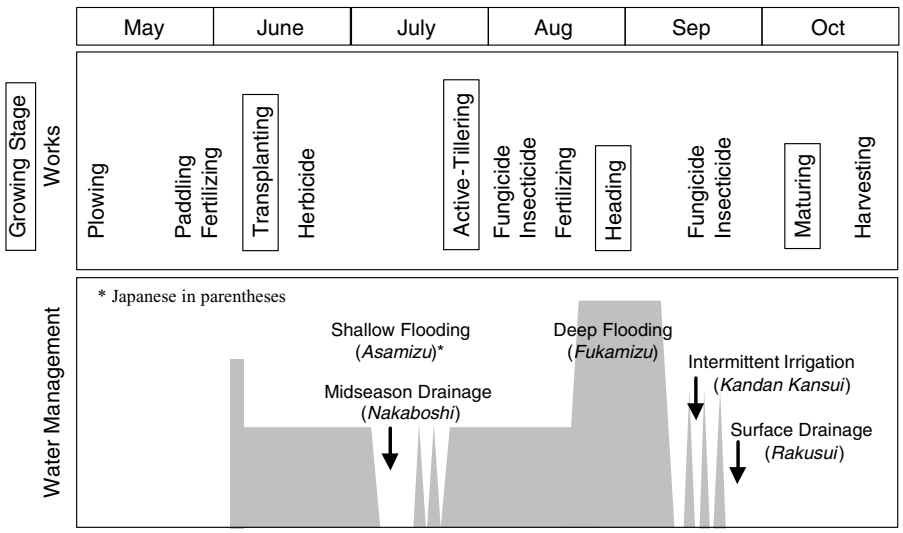


Figure 2 Typical work system and water management for rice cultivation in paddy fields

3 Study best practices

3.1 Sediment sampling

Although it is desirable to collect the sample from as many locations as possible in order to obtain a representative value, the exact number of sampling points can be determined in context with a field size or a waterway width.

Sediment samples are collected from more than four locations in a field in order to acquire samples that represent the field. Since there is a water intake and a drain in each paddy, their position should be considered in the sampling design. As shown in Figure 3, sampling points on diagonal lines of the field are recommended avoiding the area within 10 m of a water intake or a drain. In the case of the determination of a chemical in the whole plow layer (usually 25–30-cm depth), approximately 200 g of sediment column up to a 10-cm depth including surface water are collected using a sample borer for each sampling point. If the sampler is a liner installation type, as many liners should be prepared as there are samples and to be used as sample containers. If one uses one sampler for all sampling, the sampler should be washed well and rinsed with distilled water before each sampling. The leading edge of the borer should be kept sharpened with a bevel on the lower outside edge to minimize compaction of the sediment column while the borer is being pressed into the sediment. It is recommended to use a borer of diameter greater than 5 cm to avoid column compaction. The sediment columns are mixed and homogenized and a subsample is taken for laboratory analysis. To investigate the vertical distribution of a chemical for

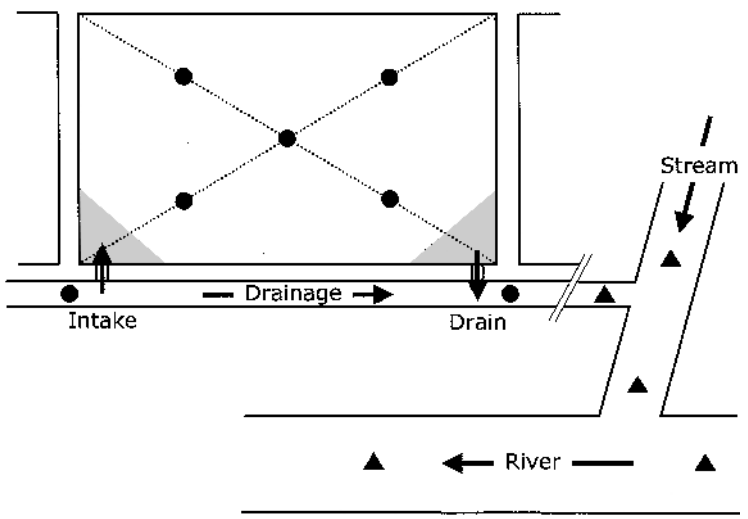

Stream
Drainage
Intake
Drain
River
Recommended sampling points for single paddy study
Area within 10 m from an intake or a drain where sampling should be avoided
Recommended sampling points for paddy field area study
(The number of samples depends on the width of waterway)

Figure 3 Recommended sampling points for a single paddy study and a paddy field area study

a leaching study, a sediment column up to the desired depth can be collected using a longer borer, then divided into parts with required depth increments. Typically sediment columns are 30 cm in length and are divided into 3–6 segments to allow the investigation of chemical mobility through the sediment profile. Flooded water on the sediment can be removed by decantation or by pushing the soil column upwards with a stick that has the same inner diameter as the borer. In this manner, the compaction ratio should be recorded for a vertical distribution study.

The method above, however, is not suitable when one needs a precise study of the vertical distribution of pesticides. Generally, the concentration of pesticides in paddy sediment is highest at the surface. Special care is required to avoid contamination with surface soil when the sediment is collected. The sediment core should be collected in two stages. First, a pipe with a diameter greater than that of the core sampler is inserted in the sediment and then water inside the pipe is removed gently with a syringe, pipet, etc. Next, a layer of surface soil (1–3 cm) is taken with a spatula or a trowel and then subsurface soil is collected with a core sampler to the desired depth; see also Figure 4.

It is useful to check for the existence of gravel or stones in the sediment beforehand since these may obstruct the insertion of a sampling borer. Putting a pipe with a length of approximately 30 cm and diameter 20–30 cm into sediment makes it easy to collect surface sediment. It is best to collect sediment with a trowel or a spatula after water inside the pipe has been removed.

For sampling sediments of adjacent water bodies, an appropriate sediment sampler such as an Ekman–Birge grab sampler for clay or loamy sediment, or a Smith–McIntyre grab sampler for sandy sediment is effective in deeper streams or rivers. These grab samplers are shown in Figure 5. Sediments in a shallow drainage can be sampled with the same method as in a paddy field. More than three locations around the sampling point decided in advance should be selected based on the width

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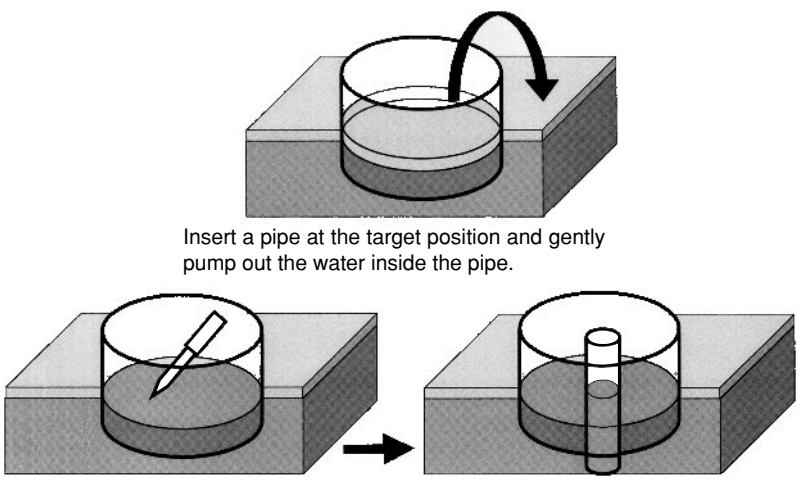

Insert a pipe at the target position and gently pump out the water inside the pipe.
Take surface sediment with a spatula, then insert a sampling borer to take cores.

Figure 4 Method of taking sediment samples in a paddy field minimizing contamination with surface sediment and water

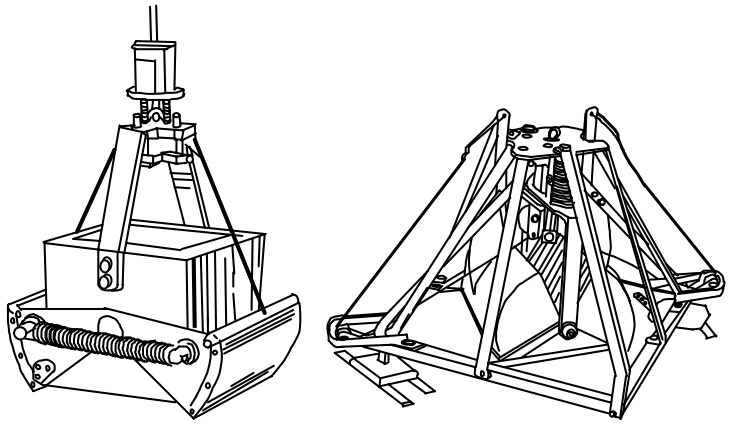


Figure 5 (a) Ekman–Birge grab sampler for clay or loamy sediments and (b) Smith–McIntyre grab sampler for sandy sediments are effective in deeper streams or rivers

of the drainage, the velocity of water flow, etc. At each sampling time, three sediment samples are taken at each sampling point. Specific sample locations should be chosen to represent the entire sampling site and should include locations in the major part of the watercourse. Since sediments may be sinks for chemicals adsorbed on soil particles, it may be necessary to analyze sediment deposits separately to determine the significance of sediment-adsorbed chemicals as a source of the chemical in the river or stream.

3.2 Water sampling

Surface water samples from a paddy field should also be collected from at least four locations. Sampling points could be chosen by the same method as sediment sampling (Figure 3). At each sampling point, approximately 200 mL of water are collected carefully from a depth of 1–3 cm into a well-washed glass bottle with a glass syringe so that bottom mud and suspended organic debris may not enter. Every bottle of water should be mixed and a subsample taken for laboratory analysis.

The required volume for the analysis of water samples from a drainage flow, stream, or river is collected from a depth of up to 50 cm at the center of a flow using an appropriate sampling bottle. A sample size of 1000 mL should be sufficient for the usual type of determination. The sampling bottle and bottles for storage and shipment should be well washed with an appropriate organic solvent and distilled water so that the sample is not contaminated, and keeping those bottles in a clean container is recommended. It is recommended that samples taken are kept below 5 °C and shipped to the laboratory as soon as possible.

During sampling, care to avoid floating materials (e.g., litter, oils, etc.) in water is necessary. Also, careful attention should be given to the collection of the water without disturbing sediments in shallow water.

If the water sample has high turbidity, it may be necessary to separate suspended solid (SS) from water before the analysis as described later. Chemicals that are hydrophobic and of low water solubility are easy to adsorb on SS.

3.3 Sample handling and shipment

3.3.1 Prevention of cross-contamination

A clean sampler should be used at different sampling points in order to prevent contamination as described earlier. A borer with a liner is recommended to minimize contamination. Using this type of sampling device, only the liner is exchanged. When a borer has to be re-used, it should be thoroughly washed and rinsed with distilled water. Other sampling instruments are dealt with in the same manner.

Special care is required to prevent contamination with surface soil when the sediment is collected to study the vertical distribution of a pesticide. The method described earlier (Section 3.1.1) is strongly recommended.

The sample water container should be made of appropriate materials to avoid adsorption of the chemical of interest on the vessel surfaces. In most cases, a glass bottle may be better than a plastic bottle. The bottle is washed with an organic solvent in advance and also washed with sample water just before sampling. The bottle should be filled to the limit with water and capped tightly with a Teflon seal to prevent contamination. The top 1-cm of water is not taken to prevent the mixing of floating materials such as oil.

3.3.2 Sample containers, labeling and shipment considerations

Sample soil or sediment is put into polyethylene bags or glass containers and sample water into bottles as mentioned above.

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A label which indicates the sample name, date and time of sampling, sampling point, sampling depth, name of sampler, etc., is attached to the container. Care should be taken that the label does not become wet and the sample information does not disappear during transportation. The general conditions at the sampling point, weather conditions, etc., at the time of the collection should be recorded on the sampling data sheet separately. It is desirable to determine and record the pH, electrical conductivity, etc., of the water at the sampling location, if possible.

An important consideration prior to sample collection is transportation and storage. Samples should be treated so as to retain the integrity of the sample from the moment of collection to the time of analysis. The physico-chemical characteristics of a sediment sample change during drying, with effects on the sorption–desorption behavior of chemicals.

Standard analytical techniques for sampling and pretreatment and analytical requirements for sediment studies are less available than for water and soil studies. To obtain meaningful results from laboratory experiments, the sediment samples should be kept in the original aqueous matrix, and analyses should be carried out immediately to minimize changes to the sample matrix due to chemical and biological processes that could occur during storage.

After removing gravel and large organic debris, the collected sediment is put into a polyethylene bag or suitable container and kept in an ice-cooled container during transportation. Collected water is transferred to a suitable bottle with a tightly sealed cap and also kept in an ice-cooled container. During transportation, cushions are packed between bottles to prevent breakage. It is best to analyze samples as soon as possible

after collection, otherwise they should be frozen at −20 °C until analysis. Care should be taken to prevent breaking of frozen glass bottles filled with water samples. A special plastic bottle (e.g., Teflon) can be used to avoid this, but may be expensive.

3.3.3 Core sectioning techniques

To investigate a vertical distribution of a chemical, a sediment column is divided into sections with appropriate thickness. The sediment column taken in a pipe should be refrigerated in an ice-cooled container, transported to the laboratory, and removed carefully on to a clean tray so that there is as little disturbance as possible to the soil core structure. In the case of a column in which there is little soil moisture and it tends to collapse, the soil should be pushed out to each required thickness and carved off. It is also possible to take a sediment column up to a 30-cm depth using a pipe that is connected to cylinders (5-cm height) with sealing tape. In this case, the sample in each 5-cm fraction can be obtained as it is, after removing the tape.

3.3.4 Importance of proper sediment/water preparation

Soil or sediment samples for the determination of agrochemicals should not be dried prior to residue analysis, whereas most soil or sediment samples are air-dried prior to analysis for inorganic nutrients such as nitrate, ammonium, metals, etc. Stones, gravel, large organic debris, etc., should be removed as much as possible since these affect the homogeneity of the sample and give rise to analytical errors. After removing large stones, etc., the sample should be passed through a 5-mm (or 2-mm, if possible) sieve using a silicon spatula in order to facilitate the passage of soil through a sieve. Sieves should be cleaned for every sample otherwise contamination may occur during the sieving process.

A water sample is usually analyzed as it is, but is filtered through a glass-fiber filter to remove SS or to analyze both water and SS separately when chemicals adsorbed on SS used to be determined for a special purpose of a study. All filtration apparatus should be washed with the sample water in order to avoid contamination.

3.3.5 Sediment moisture determination

The water content of a sample is a basic datum for calculating the value per gram of dry matter. Water content is expressed as the ratio of the mass of water present in the sample to the mass of the sample after it has been oven dried at 105 °C to a constant mass. Alternatively, the ratio of the mass of water present in the sample to the mass of the sample before oven drying is used. It is important to specify which is being used.

Also, the loss on ignition is preferably determined for a waterway sediment sample because of its high content of immature organic matter. Loss on ignition is determined with a muffle furnace in which organic matter is ignited at 600 °C for more than 2 h.

3.3.6 Extraction and cleanup techniques

Extraction and cleanup techniques for soil and water samples are described in other articles, and only comments specific to sediments are included here.

Organic solvents or mixtures of water and solvents such as acetone or water–acetone are commonly used to extract chemicals from sediment samples as for upland soil. An analysis of sediment, collected from waterways or extremely low Eh paddies, frequently requires the removal of sulfur-containing species, although there is little interference from sulfur if the sediments are in a not very reductive condition. Reduced copper and silver nitrate columns are usually used for the removal, but these procedures are not always successful. Recovery studies could be needed to confirm an interference with sulfur.

Subsequently, the determination of chemicals in the extract can be performed according to general analytical procedures that are described in other articles in this Handbook.

The concentration of agrochemicals in water samples is usually low compared with soil or crop samples except immediately after the application time. Water sample preparation may include liquid–liquid extraction, solid-phase extraction or direct analysis by reversed-phase high-performance liquid chromatography (HPLC) or liquid chromatography/mass spectrometry (LC/MS). Although there have been many advances in chromatographic analyses, some sample cleanup and concentration steps may be required to obtain the necessary specificity and quantitation limits demanded by regulatory agencies.

3.4 Quality control (QC) and quality assurance (QA)

3.4.1 Method recovery and reproducibility

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Method validation is needed to demonstrate the acceptability of the analytical method. A recovery test on a chemical being determined should be performed in order to verify the reliability of the series of analyses. Recovery studies are usually conducted by spiking untreated sediment with the target chemical at the detection limit, quantitation limit and in the range of 10–50 times the detection limit. The method is considered acceptable when the recoveries typically are greater than 70%. When the recovery is less than 70%, an improvement in the analytical methods is needed. Where this is not possible for technical reasons, then lower recovery levels may be acceptable provided that method validation has demonstrated that reproducible recoveries are obtained at a lower level of recovery. Analysis is usually done in duplicate or more, and the coefficient of variation (CV) should be less than 10% to ensure that recoveries will be consistently within the range 70–110%.

3.4.2 Techniques used to determine storage stability

Analysis should be performed as soon as possible after sample collection. When this is not possible, freeze preservation is recommended. The sample can generally be refrigerated below 5 °C, provided that the storage period is limited to a few days. Some agrochemicals may degrade during such a storage period. In order to verify the degradation of target chemicals during such storage, the stability of the chemical is examined by the addition of a known amount to the sample and storage under identical conditions. This kind of validation study is also performed to investigate the effects of transportation and shipping on stability of the target chemical in samples.

The degradation of agrochemicals during storage may result from a variety of factors such as acidic and alkaline hydrolysis, enzymatic action, etc. It is recommended that a preliminary stability study be performed for the chemical in the environmental sample. If the chemical is stable under acidic conditions, for example, samples can be stored after acidification with hydrochloric or phosphoric acid.

3.4.3 Bound residues in sediment

Some agrochemicals bind strongly to the soil component as bound residues, which cannot be extracted without vigorous extraction procedures. In this case, an acidic (e.g., hydrochloric acid, sulfuric acid) or alkaline solution (e.g., sodium hydroxide, potassium hydroxide) can be used as an extraction solvent, and also heating may be effective in improving the extraction of the residues. Analytical procedures after the extraction are the same as above, but a filtration procedure may be troublesome in some of these situations. However, these procedures are rare exceptions or are needed for specific chemicals that are stable under such harsh extraction conditions.

3.5 Data presentation and interpretation

The article on soil analysis has an extensive discussion of the kinetics on the dissipation rate. This article includes a recommendation on the data that should be reported.

3.5.1 Data presentation

Test chemicals and their use pattern information, physico-chemical properties of sediment samples, water sample quality, study field information, and climatic conditions of the study area are essential as basic information. Data concerning dissipation patterns or distributions of the chemical should be reported as those in the surface water layer, in the sediment layer, and the sum of the two. The concentration should be expressed as micrograms per kilogram for a sediment (SS also if needed) on a dry weight basis, and micrograms per liter for water.

3.5.2 Mathematical models for data interpretation

Computer-aided mathematical modeling is a useful tool to supplement monitoring studies and to evaluate the environmental fate of agrochemicals under various conditions. A simulation procedure with a mathematical model using parameters observed in the monitoring study could be helpful for the interpretation of the data obtained in the study.

Only a few models applicable to paddy field conditions have been developed. RICEWQ by Williams,[3] PADDY by Inao and Kitamura,[4] and PCPF-1 by Watanabe and Takagi [5] are useful for paddy fields. EXAMS2 by the United States Environmental Protection Agency (USEPA),[6] a surface water model, can also be used to simulate paddy fields with an appropriate model scenario and has been used for the prediction of sulfonylurea herbicide behavior in paddy fields.[7] The prediction accuracy of PADDY and PCPF-1 is high, although these models require less parameter

input than RICEWQ, because these models have been developed as models only for paddy field scenarios.

RICEWQ was the first model developed for agrochemical runoff from paddy fields, incorporating aircraft application, dissipation by drift, adhesion on leaf surfaces, and dissipation from the leaf surface in addition to the processes affecting degradation and transport in sediment and paddy water. An important parameter, desorption from sediment to paddy water, is not considered, although this is not as important as other parameters in paddy fields such as sedimentation rate, behavior of SS, etc.

PADDY focuses particularly on the granule formulation of agrochemicals in paddy fields. Considering the main processes on the basis of a compartment system, it assesses the behavior of agrochemicals. The mass-balance equations for agrochemicals in the compartments are derived from kinetic data. The main processes are dissolution of agrochemicals from the granules into paddy water, adsorption and desorption with sediment, run-off, leaching, volatilization, and degradations in sediment and water. The uptake process by plants is not considered.

PCPF-1 differs greatly from RICEWQ and PADDY in that the sediment layer is divided into an oxidative layer and a reductive layer because the 0–1-cm depth of sediment is oxidative, where most agrochemicals are adsorbed, and below 1 cm it is reductive. Agrochemical degradation can be different in the oxidative and reductive layers of the sediment. The prediction accuracy of agrochemical concentrations is improved sharply by this consideration.

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4 Conclusion

The objective of sediment and water sampling is to obtain reliable information about the behavior of agrochemicals applied to paddy fields. Errors or variability of results can occur randomly or be due to bias. The two major sources of variability are 'sediment body or water body variability' and 'measurement variability'. For the former, a statistical approach is required; the latter can be divided into sampling variability, handling, shipping and preparation variability, subsampling variability, laboratory analysis variability, and between-batch variability.[8]

All soil and sediments are naturally variable; their properties change horizontally across the landscape, although this is not as frequent in paddy fields as in upland fields. First, sufficient care should be taken with this point as it is important for sampling procedures. Typically, the error arising from field sampling is much larger than that associated with handling, preparation or analysis. Special consideration is needed for studies of paddy field and adjacent water bodies, where surface water and sediment are at the same place and collected separately. Furthermore, in such fields, surface sediments differ considerably from subsurface sediments in their performance, including adsorption of chemicals and redox potential. Hence surface sediments and subsurface sediments should be collected separately with minimum contamination when the sediment collection is done for the study of the behavior of agrochemicals such as vertical distribution, dissipation pattern, etc. Emphasis on the above points during the selection of sampling location, sampling and analysis should bring effective results.

References

1. S. Maru, *Spec. Bull. Chiba Agric. Exp. Stn.,* No.18 (1991).
2. K. Nakamura, *Bull. Saitama Agric. Exp. Stn.,* No. 46 (1993).
3. M.W. Williams, RICEWQ Users' Manual and Program Documentation, Version 1.4, Waterborne Environment, Leesburg, VA (1998).
4. K. Inao and Y. Kitamura, *Pestic. Sci.,* **55**, 38 (1999).
5. H. Watanabe and K. Takagi, *Environ. Sci. Technol.,* **21**, 1379 (2000).
6. L.A. Burns and D.M. Cline, "Exposure Analysis Modeling System: Reference Manual for EXAMS2," EPA/600/3-85/038, NTIS Publication No. PB85-228138, Environmental Protection Agency, Washington, DC (1985).
7. K.L. Armbrust, Y. Okamaoto, J. Grochulska, and A.C. Barefoot, *J. Pestic. Sci.,* **24**, 357 (1999).
8. J. Crépin and R.L. Johnson, 'Soil sampling for environmental assessment,' in "Soil Sampling and Methods of Analysis," ed. M.R. Carter, Lewis Publishers, Boca Raton, FL, pp. 5–18 (1993).

Monitoring of agrochemical residues in air

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1 Introduction

Pesticides are being applied in urban and agricultural settings at a rate of over 2 billion kilograms each year in the USA. Although these materials are applied to specific targets, such as soil, water, or plant foliage, pesticide residues can be unintentionally transported from the target site as a mixture of vapors and aerosols. Once airborne, pesticides may move downwind, where they can affect nontarget organisms such as vegetation, aquatic and terrestrial wildlife, and humans. However, the assessment of nontarget impacts of pesticides requires that pesticide transport from the source region be accurately quantified. Establishing pesticide concentrations in the ambient air, whether in the vapor or aerosol phase, requires proper selection of air samplers, sampling media, suitable field siting strategies, sampling frequencies and sampling durations. This article details various air sampling techniques and field siting strategies for monitoring pesticide residues in local or regional airsheds.

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Understanding the physical properties of the pesticide (i.e., primarily its vapor pressure) and climatic conditions are key to the selection of an appropriate field sampling and siting strategy. The distributional characteristics of airborne pesticide residues, whether vapor or aerosol, must also be considered when one is faced with the task of extracting these residues from ambient air for subsequent analysis. These characteristics determine the preferred air sampling method and procedures that must be used in the field. Furthermore, the preferred method is selected on its ability to sample, efficiently, enough material to be well above the quantitation limit of the analytical method. The fundamental goal in any air sampling design is to collect enough ambient air and to gather a sufficient number of field samples to address the underlying study objectives, whether local or regional in scope.

Handbook of Residue Analytical Methods for Agrochemicals.
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Table 1 Airborne pesticide characteristics and air sampling methods

Pesticide characteristics[a]	Appropriate sampling method
Vapor ($P^\circ > 0.1$ Pa)	Adsorbents, canisters, impingers
Vapor + aerosols ($P^\circ \approx 10^{-5}$–0.1 Pa)	Filters, adsorbents, annular denuder
Aerosols ($P^\circ < 10^{-5}$ Pa)	Filters, impactors, cyclone separators

[a] P° = compound saturation vapor pressure.[5]

2 Sample collection techniques

Vapor pressure is singly the most important physical property in determining the air sampling method of choice. The airborne distribution between vapor and aerosol in the ambient air is greatly affected by the compound's vapor pressure.[1] In the discussion that follows, sampling techniques specific to aerosol and vapor forms of pesticides will be described. For example, common methods for trapping vapors utilize adsorbents, canisters, and liquid impingers, whereas trapping aerosols and their associated pesticides may involve the use of filters and inertial samplers, such as impactors (cascade, dichotomous) and cyclone separators. However, it is expected that these specific techniques will, in practice, be used in combination to characterize both aerosols and vapor during an exposure event. For example, aerosol and vapor techniques are often used in tandem, with aerosol removal from the airstream commonly occurring first, followed by vapor removal.[2] A reverse arrangement is the annular denuder sampler that removes vapors first, by diffusion, followed by filtration, to recover aerosols, and a final adsorbent vapor trap.[3] These sampling configurations are used in recognition of the fact that there is often a distribution between aerosol and vapor for many of the semi-volatile pesticides.[4] However, no sampler arrangement can completely differentiate between aerosol and vapor, although many techniques are capable of approaching this ideal, depending on the physico-chemical properties of the analyte. A rule-of-thumb approach using compound vapor pressure can be used to estimate the physical form for airborne pesticides (Table 1).[5] Pesticides with vapor pressures greater than about 0.1 Pa will exist primarily as vapor [e.g., fumigants, *S*-ethyl dipropylthiocarbamate (EPTC)]. Pesticides with vapor pressures in the range 0.00001–0.1 Pa will lead to both vapor and aerosol (e.g., chlorinated hydrocarbons, organophosphates), and pesticides with vapor pressures less than about 0.00001 Pa (e.g., phenoxy herbicide salts, paraquat) will be found primarily in aerosols.

2.1 Chemical vapors

2.1.1 Adsorbents

The most common methods for trapping pesticide vapors from air use adsorbents. Common air sampling adsorbents include charcoal (derived from petroleum or coconut) and synthetic polymeric materials, such as cross-linked polystyrene and open-cell polyurethane foam. Charcoal has been used for the cumulative sampling of volatile

soil fumigants, such as methyl bromide,[6–8] methyl isothiocyanate (MITC),[9–15] and the halogenated refrigerants.[16,17] Charcoal contains various amounts of impurities, including metal oxides, which can successfully chemisorb airborne chemicals, many of which are gases at ambient conditions (e.g., methyl bromide and refrigerants). However, charcoal has limited applicability owing to its propensity for trapping moisture and creating strongly alkaline conditions, which will promote the hydrolysis of some susceptible chemicals (e.g., methyl bromide and MITC).[8,14,18,19] Furthermore, trapped moisture also competes with chemicals for active sites, leading to reduced trapping efficiency for many analytes. Moreover, the use of charcoal as an adsorbent is limited to relatively simple chemicals, such as methyl bromide and MITC that can be desorbed intact. More complex chemicals are often irreversibly adsorbed and can undergo chemical conversions due to heterogeneous catalysis. Polymeric materials, on the other hand, do not have these problems because they do not contain the chemically active impurities that make charcoal a relatively strong adsorbent. The trapping ability of polymeric materials is strongly affected by the volatility of the chemical analyte, since the adsorption mechanism does not involve chemisorption, but depends entirely on the weaker van der Waals dispersive and electrostatic forces. Therefore, polymeric adsorbents are limited to use for the cumulative sampling of semi-volatile compounds. A less common variant of the adsorption approach is the use of a solid matrix (e.g., glass beads, polymeric beads) coated with a chemically reactive reagent that will form a derivative with the chemical analyte during sampling. An example is Occupational Safety and Health Administration (OSHA) method 52 for sampling formaldehyde using a polystyrene support coated with 2-(hydroxymethyl)piperidine to form a stable oxazolidine derivative.[20] A similar NIOSH Method (Method 2016) uses silica gel coated with 2,4-dinitrophenylhydrazine as the derivatizing reagent to form the hydrazone.[21] This approach, however, is used in only a few special cases where derivatizing reagents of rapid reactivity are available and for which air concentrations are relatively high (e.g., $\sim$20 μg m^{-3} detection limit for formaldehyde using coated supports).

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2.1.2 Canister and bags

A sampling technique that is more commonly used in air pollution studies, but is gaining in some use for pesticide vapor sampling, employs evacuated steel canisters[22] (Figure 1a). A less costly alternative method uses gas sampling polymeric bags (Figure 1b). Although not suitable for cumulative sampling, canisters can be used for grab sampling through a rapid open–close action of the valve or time-averaged sampling by allowing a slow leak through the valve over a more prolonged period of time. In practice, the sampling valve remains open until the internal pressure of the canister equals the external pressure. For flexible, polymeric bags, two sampling options are available. Positive pressure sampling can be done, where a pump is used to 'push' a sample into the bag until it is fully inflated. The other option is to place the bag inside a vacuum chamber and pump down the chamber, allowing the bag to expand, thereby capturing a sample through a port penetrating the wall of the chamber (SKC, Eighty Four, PA, USA). In either case, the sampling rate could be rapid for grab sampling or relatively slow for time-averaged sampling.

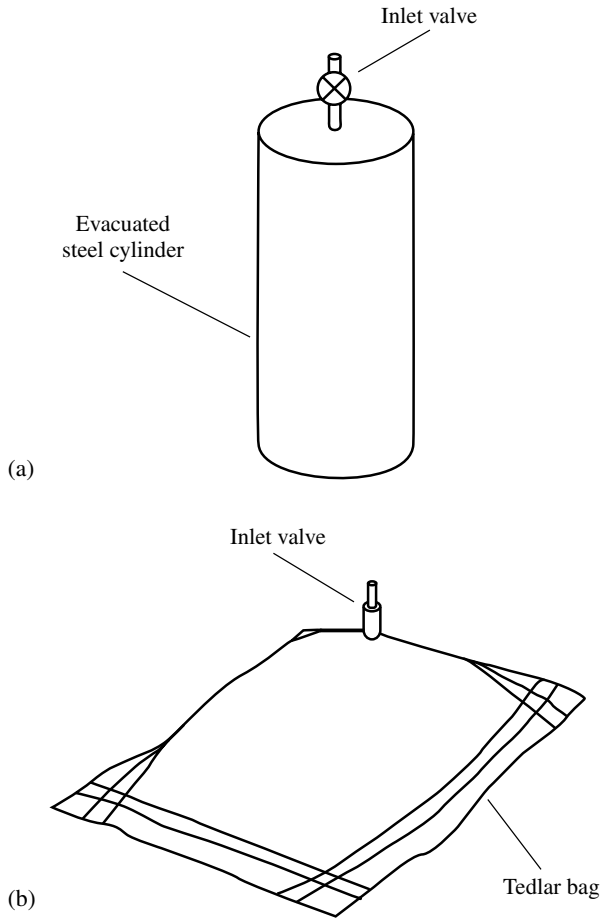


Figure 1 Schematic diagrams of (a) stainless steel air sampling canister and (b) Tedlar bag

A pump (metal bellows) can be used with canisters to meter the air sample through the valve into the evacuated canister and then to increase the internal pressure to a value greater than the external pressure before closing the valve. This feature allows sub-sampling of the canister contents without having to use a pressurization gas that would dilute the contents. Sampling bags, on the other hand, can be simply subjected to an external pressure to force the contents out into an analytical system, or a more sophisticated, computer-controlled autosampler can be used to withdraw fixed volumes of sample from the bags (SKC).

Canisters are made of highly polished stainless steel and the interior surface is passivated, usually by electropolishing or by lining with fused silica, to render it essentially chemically inert. Electropolished canisters are commonly used to sample fixed gases (e.g., CO, CO_2) and nonpolar compounds (e.g., benzene, toluene, xylene). Fused-silica lined canisters can also be used for sampling fixed gases, but they are highly recommended for sampling polar compounds and sulfur-containing compounds which may react with stainless steel. Because of passivation, canisters

can be used for relatively long-term storage of air samples, depending on the class of compound.[23] Gas sampling bags, however, are commonly made of Tedlar, a Teflon copolymer, which can be permeable to some chemicals, which results in changing sample composition over time. While sampling bags provide a cost-effective alternative to canisters, bags are unsuitable for long-term storage for most chemicals,[24] so the contents of the bag should be analyzed soon after sampling.

Unlike adsorption techniques, canisters/bags do not discriminate with regard to the chemical distribution of the air sample, since the chemical distribution of the canister/bag sample is essentially the same as for the atmosphere from which the sample was taken. Adsorbent techniques, on the other hand, are designed to trap preferentially one chemical over another. Also, adsorbent techniques often require some post-sampling workup, such as solvent desorption and concentration, that is not required with canister/bag sampling. Furthermore, canister/bag sampling avoids problems related to the interaction of the chemical analyte with the trapping medium, as can occur when charcoal is used to trap chemically sensitive volatile analytes. However, since canister/bag sampling is not a cumulative method, fairly sensitive analytical methods with relatively low detection limits are required for quantitation. In practice, to aid in detection, the entire contents of a canister/bag are often cryofocused before injection into a gas chromatograph (GC) or gas chromatograph/mass spectrometer (GC/MS) system. Additional sensitivity can be gained by operating the GC/MS in the selected-ion mode.

2.1.3 Miscellaneous methods

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A less commonly employed sampling method makes use of liquid-filled impingers, which are often filled with ethylene or hexylene glycol as the trapping medium. However, the flow rate is limited to $<25\,\mathrm{L\,min^{-1}}$ owing to aerosolization of the trapping medium at higher flow rates. In special cases where a specific analyte is being monitored, color-forming reagents can be added to the trapping liquid and the trapped analyte determined using spectrophotometric techniques.[25] However, the results require chemical concentrations in air (μg–mg $\mathrm{m^{-3}}$) that are greater than those commonly encountered in the open environment. Indicator tubes, which change color on exposure to certain classes of chemicals, are also used,[25] but the results are semiquantitative, and they are limited with regard to sensitivity (μg–mg $\mathrm{m^{-3}}$). An in situ method that shows promise for real-time determination of volatile compounds in the field is Fourier transform infrared (FTIR) spectroscopy. However, compared with the more commonly used cumulative methods, the FTIR method is currently limited with regard to sensitivity. This method has been successfully used to monitor methyl bromide after structural fumigations, where the detection limit was about 0.2 ppm (800 $\mu\mathrm{g\,m^{-3}}$).[26]

2.2 Chemicals in aerosols

The simplest approach to sampling aerosols is to use filtration or an inertial device that traps all the airborne material. This would be useful for determining the total amount

of material that could potentially be deposited in a nontarget area by dry deposition and/or in rain or fog. A typical method for determining total ambient particulates in air is filtration by high-volume (e.g., 0.5–1.0 $m^3\ min^{-1}$) air sampling through large (e.g., 10-cm diameter or 20 cm × 25 cm) glass-fiber filters.[25] These filters are capable of efficiently trapping particulates with aerodynamic diameters (ADs) down to about 1–2 μm. This approach has been typically used to determine the particulate load in a particular air basin. Smaller, more portable, lower flow versions of the described sampler are used for monitoring point sources and for personnel monitoring to aid in the assessment of exposure.

An alternative to filtration is the use of inertial samplers, represented by impactors and cyclone separators (Figure 2a and b).[25,27] Impaction, a method often used to size

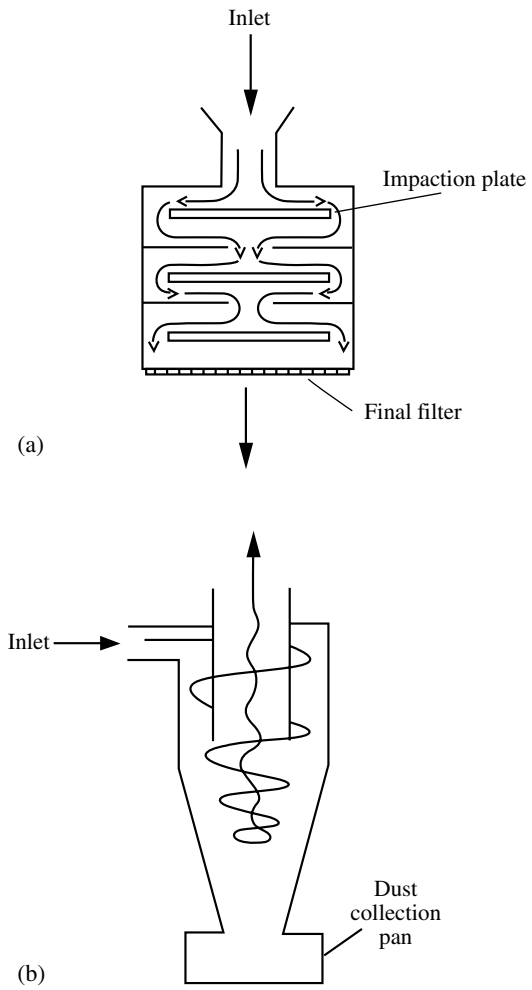


Figure 2 Examples of inertial samplers: (a) impactor (three-stage) and (b) cyclone separator

airborne particulates, can be used either to collect a particular size of particle or to separate the aerosol mix into a series of size ranges. The impactor operates by forcing an airstream through a narrow orifice, thereby imparting inertia to the aerosols by increasing the linear velocity. Aerosols of sufficient mass (i.e., momentum) will cross the streamlines and impact the collection surface. Smaller aerosols will remain in the streamlines and not be collected. In a virtual impactor, the collection surface is replaced with an open tube, the diameter of which is slightly larger than that of the orifice. To separate an aerosol mixture into multiple fractions, a cascade impactor can be employed. An impactor moves the airstream through a series of progressively smaller diameter orifices and impacts the particulates on to a surface consisting of glass fibers or, to avoid particle rebound, an oily or gummy material, to which the particles can easily adhere without being dislodged by the rapidly flowing airstream. The final stage is a filter to remove the particles too small to be trapped by impaction. The aerosol cut-size for each stage of a cascade impactor will be determined by flow rate through the sampler and by the diameter of the orifice. Instead of a critical orifice, cyclones are designed to force the airstream to impinge tangentially on the inner surface of a cylinder. As the airstream moves down the cylinder cyclonically, the cylinder tapers into a cone, increasing the angular velocity of the cyclone. This effectively imparts inertia to the aerosols, and aerosols of sufficient mass (size) will be forced to the walls of the cylinder and downward to a collection reservoir. In the cone-shaped section of the sampler, the airstream reverses direction and spirals upward and exits through a tube in the upper end of the sampler (Figure 2b). The aerosol cut-size of the cyclone is determined by the flow rate, the size of the inlet and outlet, and the size of the cylinder. A cascade of cyclones has been used to fractionate aerosols into size ranges, although a cyclone cascade cannot be made as compact as a cascade impactor.

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A common use of inertial devices is to target a particular size of the aerosol. This type of sampling is often performed to determine the respirable fraction to aid in the assessment of the impact on human/animal health of exposure to aerosols. The interest in respirable aerosols has given rise to the PM_{10} (AD = 10 μm) and $PM_{2.5}$ (AD = 2.5 μm) protocols established by the US Environmental Protection Agency (EPA).[28] The high-volume PM_{10} and $PM_{2.5}$ devices are semi-portable machines that are designed for around-the-clock operation to determine the respirable particulate load in a given air basin. These devices use a cyclone separator to divert the unwanted particle sizes from the airstream to a reservoir, allowing particulates with sizes $\leq$10 μm to be carried to the impaction surface for collection and subsequent assay. A smaller, lower flow version for personnel monitoring takes advantage of the cyclone separator technology to isolate particulates with sizes $\leq$10 μm for collection by membrane filtration.

In the special case of fogwater aerosols, dynamic samplers have been designed to condense this medium by inertial impaction on Teflon strands[29,30] or rotating stainless steel screens[31] (Figure 3a and b). The collected fogwater droplets run down the Teflon strands, aided by gravity and aerodynamic drag, through a Teflon sample trough into a collection bottle. Fogwater collected on the rotating (720 rpm) stainless-steel screens is centrifuged to the periphery, collected in a slotted aluminum tube, and drained into a collection vessel. In both cases, a large fan pulls fog-laden air through the sampling

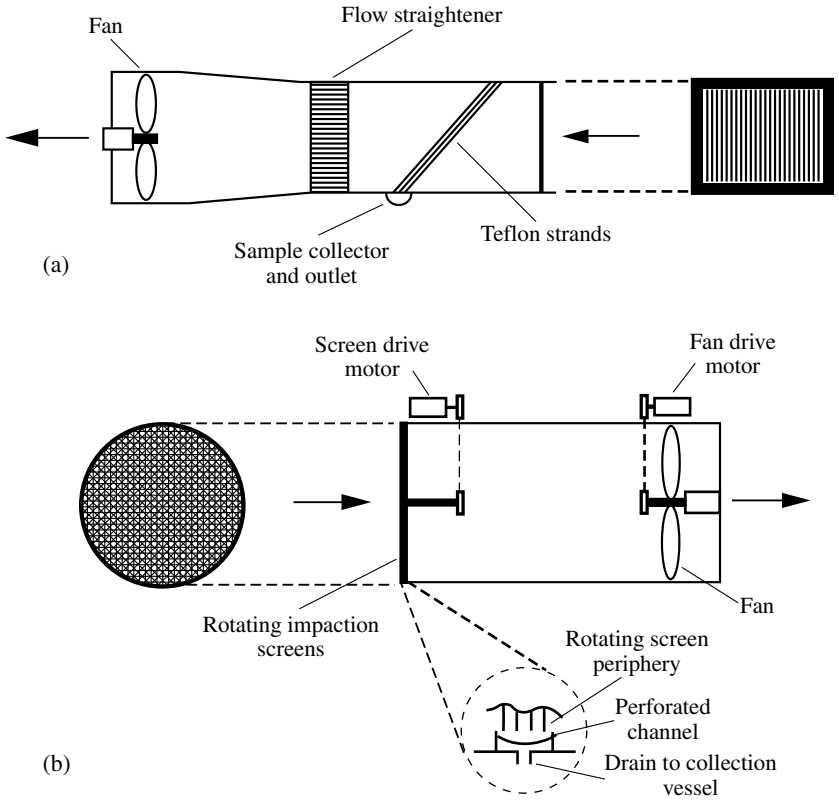
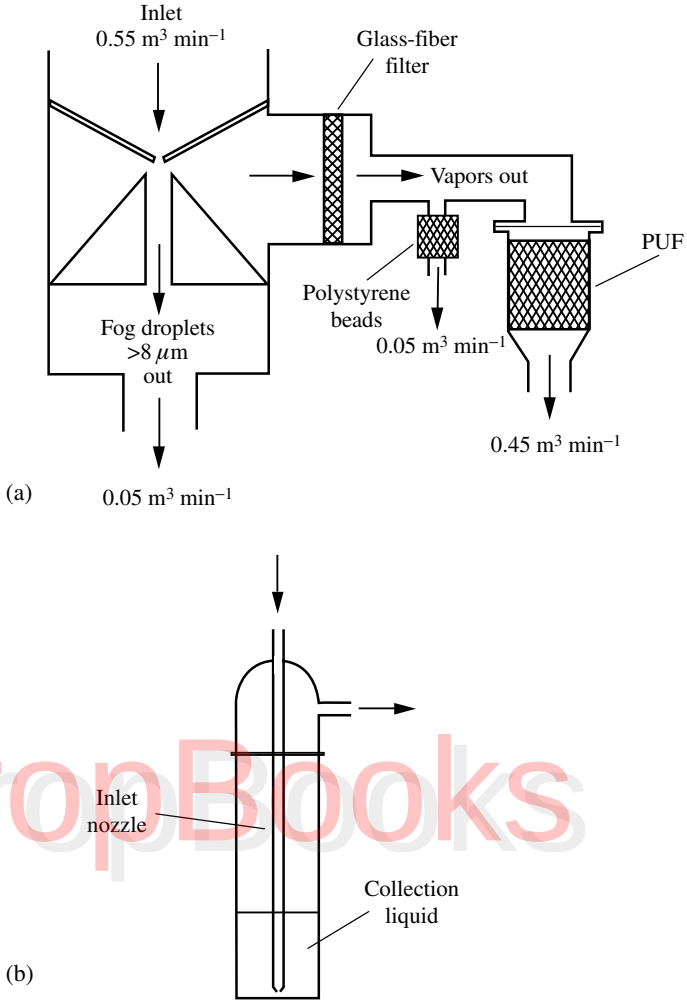


Figure 3 (a) Active strand and (b) rotating screen fogwater samplers

device. Often accompanying the fogwater sampler is a dichotomous sampler, which provides an interstitial vapor sample for comparison with the condensed fogwater. This device operates by eliminating fog droplets of size greater than about 8 μm through virtual impaction (open tube instead of an impaction surface), filtration of fog droplets of size less than 8 μm and dry aerosols, and final trapping of the vapor using an adsorbent (Figure 4a).[31]

A technique that is used to determine general exposure to airborne hazardous materials is the liquid-filled impinger, which will trap both aerosols and chemical vapors (Figure 4b). At one time, this technique was a primary air sampling method, but is now used primarily in personnel monitoring. The narrow inlet tube increases the linear velocity of the air stream, and the taper at the bottom end converts the air stream into a jet that impinges on the bottom of the cylinder. This effectively flattens the air stream into a disk for maximum interaction with the liquid. An advantage over simple filtration for particulates is the greater likelihood that semi-volatile chemicals on the particulates will be re-volatilized from the filter, whereas re-volatilization is less likely once the chemicals have been dissolved in the impinger liquid.



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Figure 4 (a) High-volume dichotomous (virtual) impactor and (b) liquid-filled impinger

3 Trapping efficiency

In general, determination of trapping efficiency for an air sampling device involves a comparison of the amount of material trapped with the original amount in the airstream. In practice, for chemical vapors trapped on adsorbents, this often means passing an airstream over a deposit of a known mass of the chemical and then passing the airstream containing volatilized chemical through the trapping medium. For whole air samplers (canisters, bags), the composition of the captured sample is compared with that of the calibration standard from which the sample was taken. For aerosols, dust feeders and nebulizers are commonly used to produce specific sizes of dust or liquid aerosols, respectively, and at known mass output rates. Aerosol sampling devices are then evaluated in terms of their ability to trap a particular size aerosol. The

following is a more detailed discussion of the determination of trapping efficiency for vapor and aerosol samplers.

3.1 Chemical vapors

3.1.1 Adsorbents

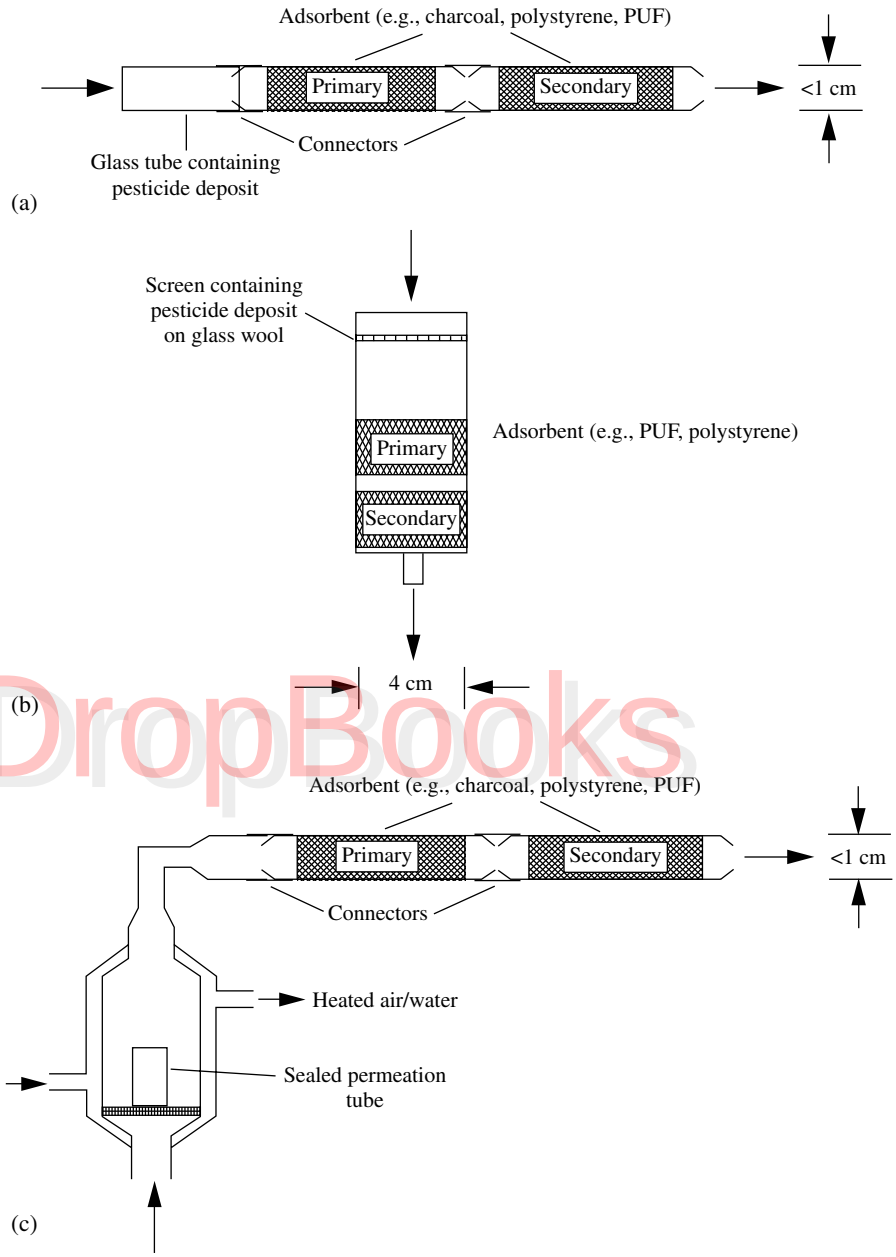
For semi-volatile compounds, a common practice is to place a deposit of a known amount of the chemical on a glass surface, flow air of known temperature and flow rate over the deposit, and direct the airstream containing the volatilized chemical through the adsorbent (Figure 5a and b). The adsorbent and original chemical deposits are then extracted with solvent and the chemical residues assayed. Trapping efficiency (%TE) is then calculated using the following expression:

$$\%\text{TE} = [(\text{mass on adsorbent})/(\text{original deposit} - \text{remaining deposit})] \times 100 \qquad (1)$$

The adsorbent also needs to be spiked directly with the chemical to determine the recovery from the adsorbent, and this recovery value can be used to adjust the recovery from the adsorbent after air sampling. For compounds with extremely low volatilities, the chemical deposit can be heated to promote volatilization and to minimize the time required for the trapping experiments.

The above approach is not entirely suitable for very volatile compounds or for compounds that are gases under ambient conditions. Deposits of very volatile compounds can volatilize quickly under typical air sampling regimes, exposing the adsorbent to unrealistically high vapor densities. Controlled metering of the volatile chemical into the airstream is required. This can be accomplished by the use of permeation tubes, consisting of sealed Teflon FEP tubes containing the volatile compound, for which permeation rates through the polymer have been established for given temperatures (Figure 5c). These tubes allow the establishment of very low concentrations of a volatile chemical in an airstream for the evaluation of an air sampling adsorbent. Already calibrated permeation tubes are available commercially for a number of chemicals or the user can prepare them. These 'home-made' permeation devices can be easily calibrated at carefully measured temperatures by weighing the device before and after an air sampling experiment. For compounds that are gases under ambient conditions, controlled metering can be accomplished by tapping into a reservoir (e.g., glass chamber, gas sampling bag) containing a mixture of air and the chemical at predetermined concentrations.

The trapping efficiency of polymeric, microporous adsorbents [e.g., polystyrene, polyurethane foam (PUF), Tenax] for compound vapors will be affected by compound vapor density (i.e., equilibrium vapor pressure). The free energy change required in the transition from the vapor state to the condensed state (e.g., on an adsorbent) is known as the adsorption potential (calories per mole), and this potential is proportional to the ratio of saturation to equilibrium vapor pressure. This means that changes in vapor density (equilibrium vapor pressure) for very volatile compounds, or for compounds that are gases under ambient conditions, can have a dramatic effect on the trapping efficiency for polymeric microporous adsorbents.



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Figure 5 Example of sampler configurations for (a) low-volume (0.1–5 L min^{-1}) and (b) high-volume (>5–100 L min^{-1}) determination of trapping efficiency for semi-volatile pesticides, and (c) low-volume (0.1–5 L min^{-1}) trapping efficiency for volatile pesticides

Table 2 Trapping efficiency related to vapor density for four common pesticides

Pesticide	Saturation vapor pressure at 25 °C (Pa)	Adsorption potential ($cal^2\ mol^{-2}$)		Trapping efficiency (%)		
		1 μg m^{-3}	1 ng m^{-3}	1 μg m^{-3}	1 ng m^{-3}	Δ (%)[a]
MITC[b]	2533	1.1×10^8	2.2×10^8	5.0	0.3	94
EPTC[c]	4.53	5.7×10^7	1.3×10^8	82	62	24
Fonofos	0.045	2.5×10^7	8.2×10^7	94	83	12
Parathion	1.22×10^{-3}	8.6×10^6	4.9×10^7	98	89	9.2

[a] Percentage difference in trapping efficiency relative to the 1 μg m^{-3} result.
[b] MITC = methyl isothiocyanate.
[c] EPTC = *S*-ethyl dipropylthiocarbamate.

The concept of adsorption potential comes from work with high-purity, synthetic microporous carbon, which relies solely on van der Waals dispersive and electrostatic forces to provide the energy for adsorption.[32–35] The polymeric microporous adsorbents that operate solely through van der Waals dispersive and electrostatic forces often cannot provide the surface potential energy needed to trap compounds that are gases under ambient conditions, and for very volatile compounds the trapping efficiency can be low for similar reasons.

To illustrate the importance of adsorption potential, four pesticides, spanning a range of vapor pressures, are compared in Table 2. Comparison is made for two vapor densities, 1 μg m^{-3} and 1 ng m^{-3}, to cover the range of pesticide concentrations often encountered in the open environment. A comparison of the adsorption potential values shows a one to two order of magnitude range for the compounds, with MITC and parathion having the greatest and least energy requirement, respectively. While these energy terms suggest that trapping would be quite different for these compounds using microporous adsorbents, a better comparison would be between trapping efficiency results. Trapping efficiency for parathion was quantitative and it changed by less than 10%, well within experimental error, for a three order of magnitude change in vapor density. A similar change in vapor density for MITC led to an order of magnitude change in the already low trapping efficiency. The other two pesticides fell between these extremes. These results indicate that trapping efficiency has an inverse relationship with compound saturation vapor pressure, and this relationship can be dramatic for fairly volatile compounds. Because of this, it is important that, when evaluating a microporous adsorbent with a fairly volatile compound, realistic environmental vapor densities be used. This can be done using permeation tubes[25] or by premixing the compound with air in a chamber, as described above. Otherwise, placing a deposit of the volatile compound in the intake manifold of an air sampling system will lead to unrealistically high vapor densities that will result in the adsorbent appearing to be a better trapping medium than is actually the case.

Based on our extensive work with pesticide air sampling, a practical saturation vapor pressure cut-off point for deciding if a microporous adsorbent would be a suitable trapping medium is approximately 0.1–0.2 Pa. Vapor pressures much above this would lead to too great an uncertainty in trapping efficiency owing to the effect of changes in vapor density. In this case, using adsorbents that interact

chemically with the analyte (e.g., charcoal) or using canisters/bags would be more suitable.

Once an adsorbent has been selected for a particular chemical, operating conditions that will lead to quantitative recovery of the chemical from an airstream for a given mass of adsorbent need to be established and adhered to as much as possible. However, it is important to find the right combination of factors to allow for some flexibility, especially with regard to ranges of vapor concentrations and temperatures. In practice, it is not always possible to anticipate all possible environmental conditions that could affect trapping efficiency. Therefore, it is generally wise as a matter of routine to use at least two sampling tubes in series, designating the first tube as the primary and using the second tube for assessing possible breakthrough. In this case, if the amount of analyte in the second tube of a two-tube sampling train is $\geq$25% of the total amount of recovered analyte (i.e., both tubes together), then the concentration in air must be reported as a 'greater than' number, reflecting the fact of some losses through the second tube.

If it becomes necessary to operate under conditions that might lead to suspected breakthrough of the chemical analyte, then a decrease in flow rate and/or an increase in the amount of adsorbent may be all that would be necessary to maintain quantitative recovery. However, if the amount of adsorbent is increased, the pressure drop through the sampler becomes a limiting factor; too great a pressure drop could result in vacuum stripping of trapped residues, especially for polymeric adsorbents that rely solely on van der Waals dispersive and electrostatic forces for trapping. It may be prudent in this case to use coarser material to help minimize pressure drop. Conversely, too low a flow rate, to minimize pressure drop, could lead to poor theoretical plates for polymeric adsorbents and, thus, poor trapping.

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As mentioned above, common adsorbents include charcoal (petroleum- and coconut-based) and polymers, such as cross-linked polystyrene (divinylbenzene copolymers, e.g., XAD-2, XAD-4) and open-cell PUF (e.g., white polyether and gray polyester). Tenax-GC, a polymer of 2,6-diphenyl-*p*-phenylene oxide, has also been used, but its relatively much greater cost has made its use in some cases prohibitive. Some examples of their usage include (1) charcoal (1–2 g, petroleum and coconut) for sampling gaseous methyl bromide at flows of $\leq$100 mL min^{-1} for periods of about 2–6 h under warm summertime and fall conditions;[6,8] (2) charcoal (1–2 g, coconut) for sampling MITC at flow rates of 1–2 L min^{-1} for periods of about 4–12 h under both winter and summertime conditions;[14,15] and (3) polystyrene/PUF for sampling semi-volatile pesticides at flow rates of about 50–1000 L min^{-1} for periods of about 2–4 h under various seasonal conditions.[36] In the last case, the amounts of polystyrene and PUF used fall in the range 10–30 g and 40–400 cm^3, respectively. For PUF, other methods recommend 30–350 cm^3 of the foam for sampling rates of about 5–200 L min^{-1} and 4–24 h, sampling duration.[22]

For the charcoal, XAD, and PUF adsorbents discussed above, solvent extraction techniques have been developed for the removal and concentration of trapped analytes. Although thermal desorption has been used with Tenax-GC in some specialized air sampling situations [primarily with sampling volatile organic compounds (EPA, Method TO-17[37])], this approach is not a viable alternative to solvent extraction for the charcoal, XAD, and PUF adsorbents. The polystyrene and PUF adsorbents are thermally unstable and the charcoal chemisorption bonding is more easily broken by

solvents that can successfully compete with the analytes for the active sites. Examples of common solvent extraction methods include (1) with charcoal, benzyl alcohol in a heated sealed glass vial for the headspace gas-chromatographic assay of released methyl bromide vapor using electron-capture detection;[7,38] ethyl acetate extraction with subsequent injection of the solvent and assay of the methyl bromide extract using capillary GC and electron-capture detection;[39,40] carbon disulfide, or a mixture with ethyl acetate, for the capillary GC assay of MITC using nitrogen–phosphorus thermionic detection;[9,14,15] and benzyl alcohol in a heated sealed glass vial for the headspace GC assay of released MITC vapor;[41] and (2) with XAD-2,4/PUF, the common method for all classes of semi-volatile pesticides is simple extraction with a suitable solvent (shaking in a flask or solvent recycling using a Soxhlet apparatus), such as ethyl acetate or acetone, or a binary mixture of solvents, such as isooctane–acetone and diethyl ether–hexane[42] (EPA, Methods TO-4A and TO-10A[43]) (the study in Ref. 42 was based on specific sampling and analysis methods documented in a series of reports available from the California Air Resources Board). Depending on the physico-chemical properties of the analyte, a single extraction by shaking in a flask may give quantitative recovery. However, multiple extractions with clean solvent may be necessary for quantitative recovery, as has been the case with the organophosphate pesticides trapped on the XAD polystyrene polymers.[42,44,45] Similarly, other methods use Soxhlet extraction, by recycling boiled and condensed solvent through the adsorbent sample, to achieve quantitative recovery of different classes of pesticides (EPA, Methods TO-4A and TO-10A[43]). Of course, whatever the extraction method, the selection of a suitable solvent will depend on a thorough knowledge of the properties of the analytes and trapping media.

3.1.2 Canisters and bags

Certification of the recovery of analytes sampled by evacuated steel canisters (i.e., trapping efficiency) is usually accomplished by allowing the canister to sample calibration standards (10 ppmv in pressurized nitrogen cylinders) diluted with humidified zero air to ppbv concentrations in a sampling manifold.[22] The evacuated canister is attached to the manifold and is allowed to sample the standards through a critical orifice, mass flow controller, or a metal bellows pump. Similarly, an evacuated Tedlar sampling bag can also be attached to the sampling manifold, either through a pump or by containing the bag in a vacuum chamber, to which a vacuum pump is attached. In either case, a GC or GC/MS probe could also be attached to the sampling manifold to perform real-time monitoring of the calibration standard concentrations during canister and bag sampling. Assay results for the whole air samples captured by the canister and bag could then be compared with the real-time results to establish recovery, or trapping efficiency. Regardless of the calibration standard, a recovery between 90 and 110% is expected for canister sampling. However, recovery for bag sampling will partly depend on how long after sampling the assay is performed. Unlike steel canisters, Tedlar bags will be somewhat permeable, depending on the chemical class of the calibration standard. In general, losses of volatile organic compounds after about 4 h will fall in the range 0–5% of original concentrations,[24] so it is imperative that the contents of a sampling bag be assayed within a few hours after sampling. Prior to sampling the calibration standards, clean canisters and bags should be used

to sample clean humidified zero air and the contents assayed for interferences and target compounds. The samplers are certified clean if the assay results show less than 0.2 ppbv interferences or targeted compounds.

Unlike the case with adsorbents, canister and bag sampling does not require the use of solvents to recover the analytes. Instead, canisters and bags are typically connected to the inlet of the GC or GC/MS analytical instrumentation where all or part of the contents are cryofocused prior to injection. If a metal bellows-type pump is used with the canister, the internal pressure can be raised above that of the ambient atmosphere during sampling. This obviously makes sub-sampling for assay fairly straightforward. However, if sampling is accomplished through a critical orifice or mass flow controller, or by simply opening the valve (grab sample), it will become necessary to pressurize the canister with an inert gas in order to sub-sample the contents for assay. However, this will result in dilution of the canister contents, requiring that a dilution factor be determined using calibration standards prior to sampling and analysis.

3.2 Aerosols

3.2.1 Aerosol generation and trapping

Samplers for trapping aerosols include relatively simple one-stage filters, cyclone separators, and impactors to multi-stage, cascade impactors. Evaluation of their trapping efficiency is accomplished by challenging these devices with monodisperse solid and liquid aerosols of various sizes. Polydisperse aerosols may be used for calibration or to simulate the actual use of equipment under controlled laboratory conditions. However, monodisperse test aerosols of known size, shape, and density are desirable because most aerosol properties depend strongly on particle size and using monodisperse aerosols best controls this variable. Tests made with a series of monodisperse aerosols, each having a different size, permit the evaluation of the effect of particle size on sampler performance.

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The various types of devices for generating aerosols include the following:[46–52] (1) nebulizers for the production of monodisperse liquid aerosols; (2) nebulizers for liquids containing dissolved or dispersed solids for the formation of monodisperse solid aerosols through the rapid evaporation of the carrier liquid; (3) nebulizers for volatile solvents containing low-volatility liquids for the production of small liquid aerosols; (4) dry dust feeders, which commonly produce polydisperse aerosols; and (5) condensation of organic vapors for both monodisperse and polydisperse aerosols. Depending on the operating conditions (i.e., pressure and flow rate), nebulizers typically produce liquid aerosols with diameters in the range 1–10 μm [mass median diameter (mass-weighted midpoint diameter)]. The polydisperse aerosols from dry dust feeders are typically smaller than about 20 μm. Aerosols with diameters less than 1 μm can be produced through the atomization of volatile solvents containing dissolved solids and volatile solvents containing suspended solid particles of known size (e.g., monodisperse polystyrene spheres) and through condensation techniques.

After aerosols are produced, various optical techniques can be used to determine their actual sizes and concentrations prior to introducing the aerosols to a sampling device. Various sizes of monodisperse aerosols can be introduced to the sampler, and its efficiency determined by measuring breakthrough using optical techniques and by

direct examination of the trapping medium. For example, a filter would be weighed before and after sampling to determine the mass of a monodisperse aerosol that was trapped for comparison with the mass introduced to the sampler. This approach could also be used to evaluate each stage of a multi-stage sampler, such as a cascade impactor, by introducing monodisperse aerosols of various sizes (mass median diameter). Final calibration of this type of sampler can be performed by introducing a characterized mixture of polydispersed aerosols and determining the distribution of the aerosol mixture among the various stages of the impactor. For samplers specifically designed to sample a particular size cut (e.g., PM_{10} and $PM_{2.5}$), size-selective devices (e.g., cyclone separators) are used upstream to remove all sizes of aerosols above the cut. In practice, the final stage of aerosol samplers consists of a filter to trap any aerosols remaining in the air stream of the sampler. In this way, aerosol samplers trap essentially 100% of the material entering the sampler. If it is suspected that the aerosols contain semi-volatile compounds, a final vapor trap, often consisting of a polymeric material (e.g., PUF, polystyrene), is added to the sampling train.

For the specific example of fogwater samplers, calibration can be accomplished by introducing characterized aqueous monodisperse aerosols of various sizes to the sampler under typical operating conditions. A nebulizer would be used under different flow rates and operating pressures to generate a range of aerosol sizes. Sampler recovery could be determined by simply comparing the volume of sampler-condensed water to the volume introduced to the sampler. For determination of trapping efficiency in the field, an optical device can be used to determine the density of the fogwater suspension.[53] Comparison would then be made between the detected volume of water in a given volume of air and the volume of fogwater collected for the same volume of air.

In all cases of aerosol sampling discussed above, the ultimate test of sampler efficiency would, of course, be the relative amount of pesticide residue recovered. Known amounts of a specific pesticide or mixture of pesticides could be introduced to the material to be aerosolized. This could be most easily accomplished using a liquid nebulizer or dust feeder by dissolving the pesticide(s) in the liquid or by coating the test dust. The liquid and dust could then be assayed to determine the actual initial levels of pesticide residues. However, depending on the physico-chemical properties of the test pesticide(s) (e.g., vapor pressure and solubility), the process of aerosolization could promote some volatilization of the pesticide(s), leading to a situation where the aerosol sampling device is exposed to a mixture of pesticides on aerosols and in the vapor. Furthermore, during the sampling process, pesticide residues may be released from the trapped aerosols. Hence it is important that a final vapor trap be included in all aerosol samplers in order to approach a pesticide mass balance.

3.2.2 Aerosol/vapor distribution of pesticides

As mentioned above, some redistribution of pesticide residues between vapor and aerosol during sampling will occur depending on the type of sampler used. Samplers that collect aerosols by filtration, followed by a vapor trap, can bias the vapor-to-aerosol ratio to higher values compared to the ratio prior to sampling.[4] This is due to volatilization of pesticide residues off trapped aerosols. The annular denuder, however, is a sampler that traps vapors first, and then the aerosols by filtration. Air containing pesticide vapor and pesticide residues on aerosols enters a series of parallel tubes or

concentric cylinders whose walls are coated with an adsorbent or high-boiling liquid. Pesticide vapors quickly diffuse to the walls as the air sample passes through the denuder, and the aerosols, whose diffusion rates are much less than the residence time of the air sample in the denuder, are carried to a filter, followed by a vapor trap. If the denuder is operated properly, where pesticide vapor is efficiently stripped from the air sample, pesticide residues on the trapped aerosols plus the residues on the vapor trap are assumed to be entirely associated with the aerosol fraction of the air sample. Compared with the first type of sampler, the denuder comes much closer to representing the true vapor-to-aerosol ratio, especially for the more volatile of the semi-volatile pesticides.[3]

4 Field sampling procedures for airborne pesticides

The fundamental purpose of field sampling is the collection of accurate and representative concentration data for the chemical(s) of interest. The proper selection of air samplers and sampling media, together with designing suitable siting strategies, sampling frequencies and sampling durations, are critical for determining pesticide concentrations in the ambient air.

The overall experimental design will also depend on the scope of the sampling program, whether local, regional, or global in nature. Localized, or field-scale, programs generally involve air sampling at near-field locations to determine pesticide air concentrations before, during, and shortly after a single point source field application. These studies are usually designed to evaluate down-wind human exposure to pesticide vapors resulting from a pesticide application. Sampling frequency is usually intense over a short timeframe, usually 1 day to 1 week. Regional assessments tend to focus on a much larger spatial scale, such as counties and states, and target more than one chemical at a time. Regional evaluations usually take place over short time spans but they may extend over an application season to monitor ambient air concentrations of agricultural pesticides that are of regional concern. Air sampling frequency is usually less intense owing to the extended experimental timeframe and longer sample collection intervals (12–24 h) that are required to detect pesticide residues from the diluted ambient air. Depending on the goal of the regional study, a considerable number of field sampling stations may be required for evaluating spatial and temporal flux of airborne pesticides.[54,55] Procedures for monitoring the global distribution of pesticides must take into account the very low concentrations of pesticides in the atmosphere. A very large volume of air must be extracted to obtain sufficient chemical to be detected by analytical instruments. High-volume air sampling devices are employed to continuously extract pesticides or persistent organic pollutant residues (POPs) from the ambient air over time intervals from days to weeks. Monitoring procedures for these persistent pesticides are not discussed in this article. However, air-sampling procedures for monitoring the global distribution of pesticides are well described in the literature.[22,56–61]

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4.1 Localized programs

Localized, or field-scale, studies generally assess pesticides drifting off-target during application or from post-application volatilization over short sampling intervals. The

monitoring objective is usually to choose representative sites that can best estimate human exposure in relatively high population areas or in areas frequented by people (e.g., schools, offices, housing tracts) in communities near agricultural areas expected to receive applications of the pesticide. Most of these studies target pesticide vapors in air. However, the occurrence and distribution of pesticides in vapor, fugitive dust, rain, and fog can also be measured for assessing exposure to humans, off-target plants and wildlife.[31,62,63] In all cases, selection of sampling media, air-sampling equipment siting, sampling frequency, and sampling durations are usually developed for estimating targeted individual pesticide concentrations in the ambient air.

4.1.1 Sampling equipment siting

Positional masts or probes capable of positioning charcoal, Amberlite XAD, or PUF adsorbent cartridges at various heights are often employed in localized field-scale assessments. General siting criteria are available for optimal mast placement for unrestricted flow of air through samplers.[64] The number, location, and vertical positioning of these sampling devices, however, will largely depend on the specific nature of the study (i.e., measuring in-field or post-application volatilization flux versus downwind transport for human, nontarget plant, or wildlife exposure assessments, etc.). Meteorological conditions, site geography, accessibility, and available resources must be considered when locating sampling sites.

An approach useful in field-sampling surrounding a treated field is illustrated in Figure 6. Designing a sampling scheme requires an understanding of the predominant wind pattern. In this example, the predominant wind flow is from the south moving

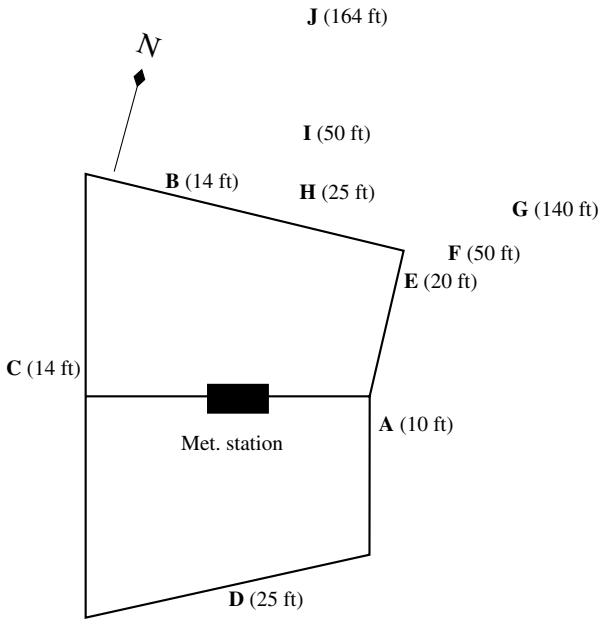


Figure 6 Illustration of siting position of air sampling masts around a treated field

northwest to northeast. The sampling stations are positioned to characterize best the pesticide plume as it volatilizes from the field. The distance in parentheses next to each station is the distance each station is located from the nearest edge of the field. Four stations, A–D, surround the field. Stations A–C are positioned to capture airborne concentrations closest to the field; these stations will have the highest concentrations. Station D is located upwind of the treated field to determine background concentrations in the airmass. It is important to have a background station in agricultural settings because there may be multiple applications taking place in nearby fields due to seasonality of applications. Stations E–J are located at increasing distances from the nearest edges of the field to determine how the pesticide concentration declines as it is swept away from the treated field. In the center of the field a meteorological station is established to record wind speed, air temperature, soil temperature, wind direction, and other important meteorological parameters necessary to characterize the movement of the pesticide.

Because of legislative mandates requiring evaluation of airborne toxins, certain State regulatory agencies have introduced uniform siting criteria useful in evaluating human exposure to agricultural pesticides in air.[42,65] For example, standardized field-siting programs are routinely conducted by California's Department of Pesticide Regulation (DPR) in conjunction with the California Air Resource Board (ARB) in counties with high historical use of agricultural pesticides. Uniform siting procedures include positioning a minimum of four mast samplers, one on each side of a field just before application. A fifth sampler is collocated at one position, normally the downwind side. Four sites are also selected that can best estimate population exposure (e.g., schools, fire stations, libraries) in a community near the agricultural field(s) expected to receive applications of the candidate pesticide. Both field and community air are monitored concurrently. A more detailed treatment on siting criteria for near-field/community human exposure assessments is described in the ARB quality assurance plan for pesticide air monitoring.[65]

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4.1.2 Sampling frequency and duration

For localized studies, pesticides tend to dissipate rapidly in air from the target application site and are typically present at highly diluted levels even shortly after application. As a result, the sampling frequency is usually intense over a short time span. A large number of sampling intervals (usually occurring within a 2-week experimental timeframe) will be required in estimating dissipation of both volatile fumigants and semi-volatile pesticides in the air after an application event. The duration in sampling time will be directly related to expected air concentrations, monitoring site distance from the target application site, prevailing meteorological conditions, and needs by the analyst for detectability of the targeted analyte. For example, low-volume pumps with flow rates up to 5 L min^{-1} can generally trap volatile fumigants that can reside in the air at high concentration levels during application and post-application. Semi-volatile compounds usually reside at more dilute concentrations in the air mass during and after application. Thus, high-volume samplers capable of sampling air from 50 to 1000 L min^{-1}, together with enough sampling media (usually 30–200-mL Amberlite XAD-2 or -4 resin or ca 40–300-cm^3 PUF plugs), will usually be required for analyte quantitation.

Table 3 Guidelines for application sampling schedule

Sample period begins	Sample duration time
Background (pre-application)	Minimum 12 h
During application	Length of application time
End of application	1 h (or up to 1 h before sunset)
1 h post-application	2 h (or up to 1 h before sunset)
3 h post-application	3 h (or up to 1 h before sunset)
6 h post-application	6 h (or up to 1 h before sunset)
1 h before sunset	Overnight (until 1 h before sunrise)
1 h after sunrise	Daytime (until 1 h before sunset)
1 h before sunset	Overnight (until 1 h after sunrise)
1 h after sunrise	24 h (until 1 h after sunrise)

Reproduced by permission from 'Quality Assurance Plan for Pesticide Air Monitoring', California Air Resources Board (1999).

Table 3 provides general guidelines used in toxic air assessments in California for sampling common agricultural pesticides in ambient air at near-field sampling monitors before, during, and shortly after a field application. For communities that are near the site of the candidate pesticide application, concurrent ambient air samples are taken over durations of 24 h and are collected 4 days per week for a period of 4 weeks.

4.2 Regional field procedures

Regional transport usually occurs over a range of tens to hundreds of miles from the application region. Field sampling procedures must be appropriately designed to account for a variety of airborne pesticides and their reactive and sometimes toxic by-products that can be distributed among air, aerosol, or particulates at highly dilute atmospheric concentrations. Although these studies provide less specific information than localized studies, regional air assessments are in many ways similar in that field and analytical procedures are often selected for a series of known pesticides. Also, knowledge of the use patterns of agricultural pesticides and climatic conditions in the region remains important in timing of field sampling and devising suitable air sampling methodologies.

4.2.1 Sampling equipment siting

Positional masts or probes that are similar to those used in localized assessments are often employed in conducting regional assessments. Sampling sites are often located in more remote areas that do not have electricity. Consequently, electrical generators are often required to provide power to the high-volume air samplers. The number of sites will vary from a few for qualitative evaluations of airborne pesticides in a region to many for estimating air movement and deposition in a basin-wide geographical region. For example, one to a few monitoring locations situated in pristine areas well away from agricultural regions can provide very useful ecological exposure data on unintentional regional transport of airborne pesticides.[44,62,66] Estimating regional

source contributions to watersheds and/or estuaries will likely require a greater number of sampling sites. For example, McConnell *et al.*[67] were able to estimate seasonal atmospheric loading of chlorpyrifos to the Chesapeake Bay based on collections taken from eight stations positioned down the main-stem of this bay. Still more monitoring sites are required when the goal is to model spatial and temporal pesticide movement and deposition in geographical regions of high pesticide use. In one of the more comprehensive regional siting programs, between 1970 and 1972 Kutz *et al.*[54] collected 24-h air samples from 26 sites positioned in 16 States where pesticide concentrations were likely to be detected in the ambient air, as part of a national pesticide monitoring program. Similar monitoring programs with multiple concurrent sampling sites have also been employed for the assessment of volatile and semi-volatile phenoxy herbicide formulations in the Pacific Northwest impacting grape production.[68] Even in these comprehensive regional studies, only general distributional trends with regard to the movement of insecticides could be ascertained owing to the nonpoint nature of sources, limited number of regional sites, and specificity of the analytical method(s) needed for the pesticides encountered.

4.2.2 Sampling frequency and duration

To account for substantial dilution, sampling large volumes of air taken over extended time intervals up to 24 h or longer are usually required for trapping enough pesticide needed in residue determinations. High-volume two-stage air samplers capable of pulling air through a porous glass-fiber filter to retain particulates and then through an adsorbent (PUF or XAD resin) to trap gaseous semi-volatile pesticides are often used in regional assessments. Flow rates can range from 50 to $>1000\,\mathrm{L\,min^{-1}}$ and are set according to anticipated analytical detectability requirements of the study and to minimize breakthrough. The EPA also provides useful interval sampling guidelines for various airborne pesticide groups when using two-stage glass-fiber filter/PUF assemblies.[22]

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Although only a few samples may be taken on a daily or weekly basis from each sampling site, the total number of samples may be substantial since experimental timeframes may extend over many months. For example, in the comprehensive 2-year national pesticide monitoring program study by Kutz *et al.*,[54] nearly 2500 samples were collected which individually assessed over 40 individual pesticides and reaction by-products per sample.

A regional air monitoring sampling scheme is illustrated in Figure 7. This study was conducted in 1995 to determine the ambient concentrations of methyl bromide in the Salinas Valley of Central California.[69] The Salinas Valley is a coastal valley approximately 18 miles wide and 47 miles in length, oriented in a NW–SE direction. It is bounded to the east by the Gabilan mountain range and the Sierra de Salinas Mountains to the West. The typical wind pattern is a northwest flow during the day and a southeast flow during the evening. Methyl bromide is predominantly used in strawberry fields in and around the area of Salinas, so in this case Salinas was considered the source of emission. Air samples were collected at 11 sites throughout the valley and surrounding hills. The sampling site located on the coast of Monterey was used as a background site; any concentration of methyl bromide at this site during on-shore breezes would indicate the ocean as a source.

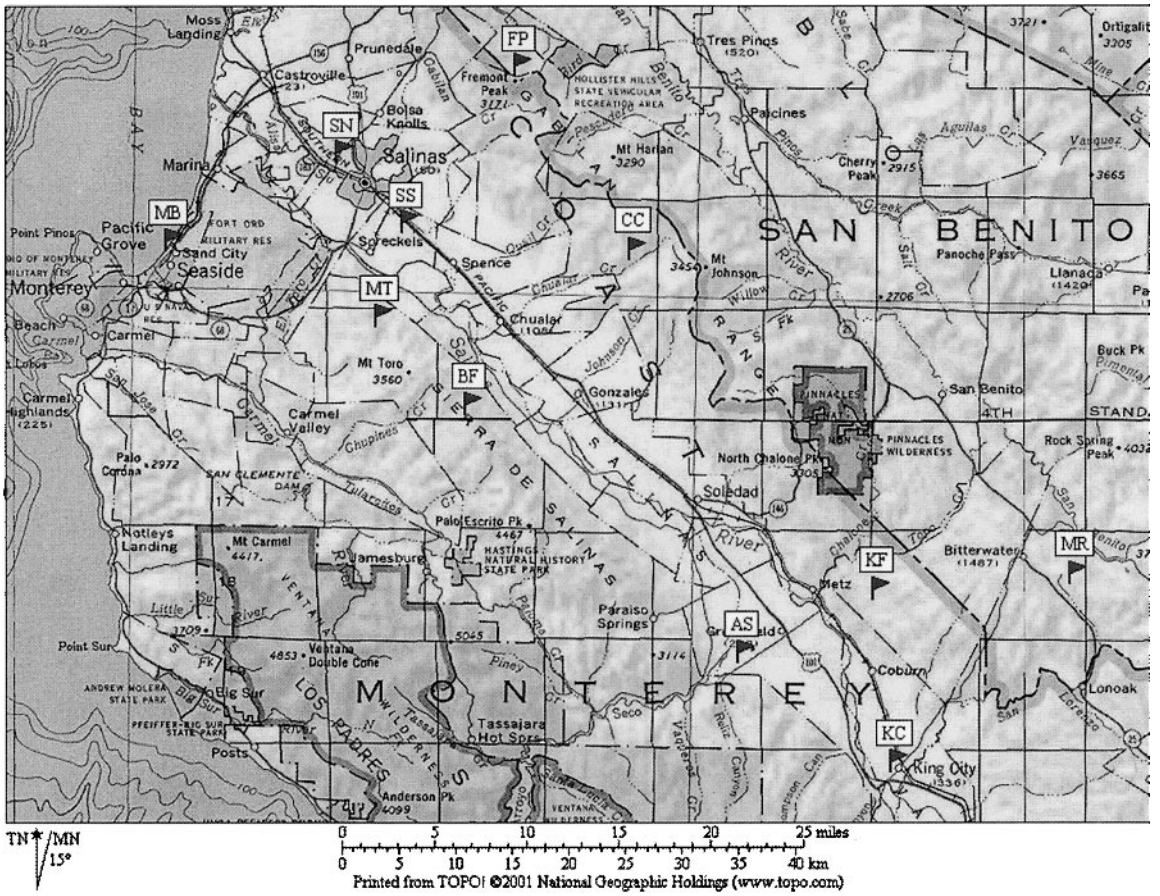


Figure 7 Illustration of air sampling station locations (▶) in a regional study. Map created with TOPO! ©2001 National Geographic Holdings

4.3 QA/QC considerations

Data generated by environmental and biomedical studies are required to stand up to scrutiny in a court of law, owing to the growing possibility of litigation. Furthermore, performing an air sampling study using good science may not guarantee the overall quality or constructibility of the generated data. Therefore, investigators now formulate and implement procedures that guarantee the quality and reliability of the project data. Procedures for ensuring the quality of data related to air sampling include (1) calibration of air sampling equipment and analytical instrumentation to attain accuracy, (2) replication to establish precision limits, and (3) determination of the stability of the analytes during sampling, sample workup for analysis, and storage.

The air sampler manufacturer, who will also supply certification, often performs calibration of certain kinds of air sampling equipment. Air pumps and blowers will always require calibration when used. This involves the use of flow meters certified for accuracy by the manufacturer. In the same way, analytical instrumentation will

require periodic calibration during an assay. This is accomplished by the use of pure reference standards of the target analytes prepared at various concentrations of experimental interest to generate multipoint calibration curves that are often repeated. It is recommended that new calibration curves be routinely generated to account for the possibility of instrument 'drift' due to 'aging' of the analytical column and detector. In addition, 'spot checks' should be made every 2–5 samples (i.e., depending on instrument baseline stability) using suitable calibration standards. If reference standards are obtained from commercial sources, suppliers will often certify purity. Otherwise, purity will need to be established using standard analytical techniques (e.g., GC/MS) with documentation. If reference standards are not available and synthesis is required, preparation and purification of the needed reference analyte(s) must be carefully documented. Once the reference standards are at hand, they will need to be properly labeled and stored under conditions that will maintain stability (e.g., in a refrigerator/freezer away from light). Even so, an expiration date should be assigned, but one that is reasonable with regard to the physical and chemical properties of the analyte(s). Pure primary standards, from which secondary dilute standards will be prepared, should be maintained in a freezer and have an assigned expiration date. The secondary standards, which will be used to calibrate the analytical instrumentation and determine analyte stability and recovery, should have assigned expiration dates, but ones that are more current owing to frequent handling.

As indicated earlier, background ambient air sampling should be performed at each of the individual sampling stations before a known application event, if possible. Flow controllers (rotameter, electronic flow controller, or critical orifice) should be calibrated in the field against a reference standard prior to a monitoring period. Replication in the field involves taking collocated samples during each sampling period. Assay results for collocated samples will give the precision for sampling under field conditions, and concentrations in air will be the averages of the assay results, with standard deviations reflecting both systematic and random errors. For proper statistics, at least three, and preferably more, collocated samples should be taken during a particular sampling period.

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4.3.1 Laboratory, trip and field spikes

All fortified (spiked) matrix samples are prepared in the laboratory at the same concentration. Laboratory spikes are immediately put into cold storage. Trip and field spikes are kept cold and sent to the field. The trip spikes will accompany sample shipments. The field spikes are stored and transported in the same manner as the trip spikes. When practical, air should be pulled through field spikes in the same manner as actual field samples being taken at the time of the study.

Analyte stability is also of concern during sampling, sample workup, and storage. Stability in the field during sampling can be assessed by using reference standards to spike an air sampler far removed from a specific source or to spike an air sampler at levels well in excess of expected environmental levels. In this way, it is possible to determine if conversion of an analyte under field conditions is an artifact of sampling, a result of environmental conditions and physico-chemical properties, or a combination of all.[70] The stability of solvent-extracted laboratory samples during the workup process can be evaluated by simply comparing expected percentage recoveries of

a laboratory-fortified extract against a reference standard of known concentration. Stability during freezer storage can be assessed by including laboratory-spiked matrix samples along with the actual collected field samples. Over the course of time as the field samples are assayed, several freezer spikes can be removed from time to time for assay also, leaving several spikes in the freezer to be assayed after all the field samples have been processed. In this way, it is possible to determine the time course of analyte decline during storage. Freezer blanks, consisting of clean trapping medium, can also be included to assess the stability of the medium during storage and the possibility of the buildup of freezer-related contaminants over time. An exception to the freezer approach involves the use of canisters, which are usually stored at room temperature. It is a general practice, for quality assurance (QA)/quality control (QC) considerations, to assay 10% of the canisters a second time. To assess long-term stability of analytes, sub-sampling of single canisters over a period of months may be required.

For all of the QA/QC-related activities just described, thorough documentation is of crucial importance. Standard operating procedures (SOPs) should be prepared well ahead of sampling describing and documenting (1) the calibration and operation of specific sampling equipment, (2) the preparation, storage, shipment, and handling of samples, (3) the calibration and operation of analytical instrumentation, and (4) all aspects of data recording and processing, including computer hardware and software used. The SOPs should include specific stepwise, clearly written instructions and be developed by the laboratory personnel conducting the work. At a minimum, documentation should include field notebooks and logs, equipment/instrumentation operation manuals and maintenance logs, sample chain-of-custody forms, sample receiving and storage/archival forms and logs, sample handling logs, and final reports. All documentation must be dated and signed at the time the documentation is created.

5 Summary

Airborne residues of pesticides will occur as vapor and in aerosol form. The distribution between vapor and aerosol will be greatly affected by compound vapor pressure. This property will also be one of the important factors in determining the air sampling method of choice. For those pesticides that are mostly or solely in vapor form, the sampling options available include adsorbents and canisters/bags. The performance of polymeric adsorbents that rely on surface electrostatic potential to trap pesticides will be greatly affected by compound vapor pressure (i.e., adsorption potential). Pesticides with vapor pressures somewhat greater than about 0.1–0.2 Pa will not be efficiently trapped by polymeric adsorbents. In this case, chemisorption on charcoal or inert solid supports coated with derivatizing reagents would be a more reasonable choice. An alternative choice for volatile pesticides would involve the use of canisters/bags. Unlike adsorbents, which are necessarily cumulative, canister/bags do not concentrate the analyte, but instead are 'whole air' samplers. However, canisters/bags can be used for grab sampling and also for time-weighted sampling.

For aerosols of nonvolatile pesticides (e.g., paraquat) and aerosols containing pesticides, sampling methods consisting of filtration and employing inertial samplers

(impactors, cyclones) are available. If there is a chance that pesticides on aerosols can volatilize to some extent, then the aerosol sampler is followed by a vapor trap, usually consisting of a polymeric adsorbent. If the distribution between aerosol and vapor is important, then annular denuders can be used first to trap vapors by diffusion, and then to trap the aerosol by filtration, followed by a vapor trap to collect aerosol-associated residues.

For developing suitable localized and regional evaluations, the proper selection of air samplers and sampling media, together with designing suitable siting strategies, sampling frequencies, and sampling durations, will be critical in establishing pesticide concentrations in the ambient air. Considering the distribution of the pesticide among vapor and aerosol phases at equilibrium becomes especially important for regional assessments when developing suitable sampling procedures, whether single-stage adsorbent or multi-stage aerosol–vapor air sampling devices. There still continues to be limited guidance made available to local, State and Federal agencies and other research institutions on field procedures for sampling pesticides from ambient air. This limited guidance is directly associated with the complexity in developing standardized sampling procedures for an array of pesticides with different methods of field application, types of formulation, and diverse physico-chemical properties. As a result, the lack of consistency in sampling methodologies, sampling site placement, collection times, and sampling durations will continue to make comparisons of residue results difficult to interpret. The on-going efforts by the EPA in preparing a compendium of analytical methods for sampling airborne pesticides are commendable.[22] The efforts of the California ARB in providing procedural guidelines for localized near-field sampling are also to be commended and should be useful for the construction of verifiable procedures for future local and regional air assessments.[42]

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Owing to increasing local and regional public concern and need for data comparability, greater efforts should continue to be directed at establishing more uniform sampling procedures for pesticides in air. Regardless of the field sampling procedure and air sampling method that one chooses, the overarching concern should always be quality of the data. Assurance of quality can be met by instituting sound and verifiable laboratory practices at the start of any air sampling program. This, for the most part, involves having SOPs in place at the start for all activities (e.g., equipment calibration, sampling, sample handling, assay, storage, stability, etc.) and developing a clear plan of action or protocol. Perhaps the most important practice is to ensure study construction through rigorous documentation that can be verified by an independent quality assurance unit. In combination with the SOPs, protocol, documentation, and verification of all activities will go far to provide defensible data and conclusions of the program and lead to a product that will be able to stand on its own.

References

1. M.S. Majewski and P.D. Capel, in 'Pesticides in the Atmosphere: Distribution, Trends, and Governing Factors,' ed. R.J. Gilliol, Vol. 1, 'Pesticides in the Hydrologic System,' Ann Arbor Press, Ann Arbor, MI (1995).
2. R.G. Lewis and S.M. Gordon, 'Sampling for organic chemicals in air,' in 'Principles of Environmental Sampling,' ed. L.H. Keith, American Chemical Society, Washington, DC, Chapt. 23 (1996).

3. A.J. Peters, D.A. Lane, L.A. Gundel, G.L. Northcott, and K.C. Jones, *Environ. Sci. Technol.,* **34**, 5001 (2000).
4. T.F. Bidleman, *Environ. Sci. Technol.,* **22**, 361 (1988).
5. J.N. Seiber and J.E. Woodrow, 'Airborne residues and human exposure,' in "Determination and Assessment of Pesticide Exposure," ed. M. Siewierski, Studies in Environmental Science 24, Elsevier, New York, pp. 133–146 (1984).
6. J.N. Seiber, J.E. Woodrow, P.S. Honaganahalli, J.S. LeNoir, and K.C. Dowling, 'Flux, dispersion characteristics, and sinks for airborne methyl bromide downwind of a treated agricultural field,' in "Fumigants: Environmental Fate, Exposure, and Analysis," ed. J.N. Seiber, J.A. Knuteson, J.E. Woodrow, N.L. Wolfe, M.V. Yates, and S.R. Yates, ACS Symposium Series No. 652, American Chemical Society, Washington, DC, pp. 154–177 (1997).
7. J.E. Woodrow, P.S. Honaganahalli, and J.N. Seiber, 'Determination of methyl bromide in air resulting from pest control fumigations,' in "Fumigants: Environmental Fate, Exposure, and Analysis," ed. J.N. Seiber, J.A. Knuteson, J.E. Woodrow, N.L. Wolfe, M.V. Yates, and S.R. Yates, ACS Symposium Series No. 652, American Chemical Society, Washington, DC, pp. 189–201 (1997).
8. J.E. Woodrow, J.S. LeNoir, and J.N. Seiber, *Chemosphere,* **35**, 2543 (1997).
9. A. Collina and P. Maini, *Bull. Environ. Contam. Toxicol.,* **22**, 400 (1979).
10. L.G. Tuinstra, W.A. Traag, and A.H. Roos, *J. High Resolut. Chromatogr. Chromatogr. Communi.*, **11**, 106 (1988).
11. F. van den Berg, *Atmos. Environ., Part A,* **27**, 63 (1993).
12. CDPR, Air Monitoring for Methyl Isothiocyanate During a Sprinkler Application of Metam-Sodium, Report EH 94-02, California Department of Pesticide Regulation, Sacramento, CA (1994).
13. F. van den Berg, A.H. Roos, L.G.M.T. Tuinstra, and M. Leistra, *Water Air Soil Pollut.,* **78**, 247 (1994).
14. J.E. Woodrow, J.S. LeNoir, J.N. Seiber, T. Dinoff, and R.I. Krieger, Determination of MITC in Air Downwind of Fields Treated with Metam Sodium by Drip Irrigation, Final Report to the Metam Sodium Task Force, University of Nevada, Reno, NV and University of California, Riverside, CA (1998).
15. J.N. Seiber, J.E. Woodrow, R.I. Krieger, and T. Dinoff, Determination of Ambient MITC Residues in Indoor and Outdoor Air in Townships near Fields Treated with Metam Sodium, Final Report to the Metam Sodium Task Force, University of Nevada, Reno, NV and University of California, Riverside, CA (1999).
16. NIOSH, 'Method 1006 (Freon 11),' in "Manual of Analytical Methods (NMAM)," ed. M.E. Cassinelli and P.F. O'Conner, fourth edition, DHHS (NIOSH) Publication 94-113, Department of Health and Human Services, Washington, DC (1994).
17. OSHA, 'Method 113 (Freon 141b, Freon 113),' **http://www.osha-sic.gov/dts/sltc/methods/organic/org113/org113.html** (1998).
18. J. Gan, M.A. Anderson, M.V. Yates, W.F. Spencer, and S.R. Yates, *J. Agric. Food Chem.,* **43**, 1361 (1995).
19. P.M. Jeffers and N.L. Wolfe, 'Hydrolysis of methyl bromide, ethyl bromide, chloropicrin, 1,4-dichloro-2-butene, and other halogenated hydrocarbons,' in "Fumigants: Environmental Fate, Exposure, and Analysis," ed. J.N. Seiber, J.A. Knuteson, J.E. Woodrow, N.L. Wolfe, M.V. Yates, and S.R. Yates, ACS Symposium Series No. 652, American Chemical Society, Washington, DC, pp. 32–41 (1997).
20. OSHA, 'Method 52 (formaldehyde),' in Analytical Methods Manual, US Department of Labor, Occupational Safety and Health Administration, OSHA Analytical Laboratory, Salt Lake City, UT (1985).
21. NIOSH, 'Method 2016 (formaldehyde),' in "Manual of Analytical Methods (NMAM)," ed. M.E. Cassinelli and P.F. O'Conner, fourth edition, DHHS (NIOSH) Publication 94-113, Department of Health and Human Services, Washington, DC (1994).
22. EPA, 'Compendium of Methods for Toxic Organic Air Pollutants,' Methods TO-1 through TO-17, EPA/625/SR-96/010b, US Environmental Protection Agency, Washington, DC (1999).
23. D.A. Brymer, L.D. Ogle, C.J. Jones, and D.L. Lewis, *Environ. Sci. Technol.,* **30**, 188 (1996).
24. J.C. Posner and W.J. Woodfin, *Appl. Ind. Hyg.,* **1**, 163 (1986).

25. J.P. Lodge, Jr. (ed.), 'Methods of Air Sampling and Analysis,' third edition, Lewis, Chelsea, MI (1989).
26. H.W. Biermann, 'Time-resolved air monitoring using Fourier transform infrared spectroscopy,' in "Fumigants: Environmental Fate, Exposure, and Analysis, ed. J.N. Seiber, J.A. Knuteson, J.E. Woodrow, N.L. Wolfe, M.V. Yates, and S.R. Yates, ACS Symposium Series No. 652, American Chemical Society, Washington, DC, pp. 202–211 (1997).
27. K. Willeke and P.A. Baron (eds), 'Aerosol Measurement: Principles, Techniques, and Applications,' Van Nostrand Reinhold, New York (1993).
28. EPA, 'National ambient air quality standards for particulate matter (40 CFR 50, 53, 58),' *Fed. Regist.,* **62**(138), 1–102, July 18 (1997).
29. B.C. Daube, Jr, R.C. Flagan, and M.R. Hoffmann, 'Active cloudwater collector,' US Pat. 4 697 462 (1987).
30. J.L. Collett, Jr, B.C. Daube, Jr, J.W. Munger, and M.R. Hoffmann, *Atmos. Environ. Part A,* **24**, 1684 (1990).
31. D.E. Glotfelty, J.N. Seiber, and L.A. Liljedahl, 'Pesticides and other organics in fog,' in "Measurement of Toxic Air Pollutants, Proceedings of the 1986 EPA/APCA Symposium," April 27–30, 1986, Air Pollution Control Association/EPA, Raleigh, NC, pp. 168–175 (1987).
32. M.M. Dubinin and E.D. Zaverina, *Zhru. Fiz. Kim.,* **23**, 1129 (1949).
33. L.A. Jonas and J.A. Rehrmann, *Carbon,* **10**, 657 (1972).
34. L.A. Jonas and J.A. Rehrmann, *Carbon,* **11**, 59 (1973).
35. P.J. Reucroft, W.H. Simpson, and L.A. Jonas, *J. Phys. Chem.,* **75**, 3526 (1971).
36. D.A. Kurtz (ed.), 'Long Range Transport of Pesticides,' Lewis, Chelsea, MI (1990).
37. EPA, 'Compendium of Methods for Toxic Organic Air Pollutants,' Method TO-17, EPA/625/R-96/010b, US Environmental Protection Agency, Washington, DC (1999).
38. J.E. Woodrow, M.M. McChesney, and J.N. Seiber, *Anal. Chem.,* **60**, 509 (1988).
39. L.J. Ross, B. Johnson, K.D. Kim, and J. Hsu, *J. Environ. Qual.,* **25**, 885 (1996).
40. H.W. Biermann and T. Barry, 'Evaluation of Charcoal Tube and SUMMA Canister Recoveries for Methyl Bromide Air Sampling,' Report EH 9902, California Environmental Protection Agency, Department of Pesticide Regulation, Sacramento, CA (1999).
41. J. Gan, S.R. Yates, W.F. Spencer, and M.V. Yates, *J. Chromatogr. A,* **684**, 121 (1994).
42. L.W. Baker, D.L. Fitzell, J.N. Seiber, T.R. Parker, T. Shibamoto, M.W. Poore, K.E. Longley, R.P. Tomlin, R. Propper, and D.W. Duncan, *Environ. Sci. Technol.,* **30**, 1365 (1996).
43. EPA, 'Compendium of Methods for Toxic Organic Air Pollutants,' Methods TO-4A and TO-10A, EPA/625/R-96/010b, US Environmental Protection Agency, Washington, DC (1999).
44. J.M. Zabik and J.N. Seiber, *J. Environ. Qual.,* **22**, 80 (1993).
45. L.S. Aston and J.N. Seiber, *J. Environ. Qual.,* **26**, 1483 (1997).
46. K.R. May, *J. Aerosol Sci.,* **4**, 235 (1973).
47. R.A. Gussman, *Am. Ind. Hyg. Assoc. J.,* **45**, No. 3, B8 (1984).
48. B.M. Wright, *J. Sci. Instrum.,* **27**, 12 (1950).
49. P.L. Magill, F.R. Holden, and C. Ackley (eds), 'Air Pollution Handbook,' McGraw-Hill, New York (1956).
50. R. Dennis (ed.) 'Handbook on Aerosols,' TID No. 26608, National Technical Information Service, Washington, DC (1976).
51. K. Willeke (ed.), 'Generation of Aerosols and Facilities for Exposure Experiments,' Ann Arbor Science, Ann Arbor, MI (1980).
52. W.C. Hinds, Aerosol Technology: Properties, Behavior, and Measurement of Airborne Particles. John Wiley & Sons, New York (1982).
53. R.K.A.M. Mallant and G.P.A. Kos, *Aerosol. Sci. Technol.,* **13**, 196 (1990).
54. F.W. Kutz, A.R. Yobs, and S.C. Yang, 'National pesticide monitoring programs,' in "Air Pollution from Pesticides and Agricultural Processes," ed. R.E. Lee, Jr, CRC Press, Cleveland, OH, pp. 95–136 (1976).
55. D.A. Goolsby, E.M. Thuman, M.L. Pommes, and W.A. Battaglin, 'Temporal and geographic distribution of herbicides in precipitation in the Midwest and Northeast United States, 1990–1991,' in 'Proceedings of the Fourth National Pesticide Conference,' Richmond, VA, November 1–3, 1993, pp. 20–22 (1994).

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56. B.R. Hillery, M.F. Simcik, H. Basu, R.M. Hoff, W. Strachan, D. Burniston, C.H. Chan, K.A. Brice, C.W. Sweet, and R. Hites, *Environ. Sci. Technol.,* **32**, 2216 (1998).
57. J. Haugen, F. Wania, and Y. Lei, *Environ. Sci. Technol.,* **33**, 2340 (1999).
58. L. McConnell, J. Kuchlick, T. Bidleman, G. Ivanov, and S. Chernyak, *Environ. Sci. Technol.,* **30**, 2975 (1996).
59. M. Oehme, J.-E. Haugen, and M. Schlabach, *Environ. Sci. Technol.,* **30**, 2294 (1996).
60. H. Iwata, S. Tanabe, N. Sakia, and R. Tatsukawa, *Environ. Sci. Technol.,* **27**, 1080 (1993).
61. R. Taksukawa, Y. Yamaguchi, M. Kawano, N. Kannan, and S. Tababe, 'Global monitoring of organochlorine insecticides—an eleven year case study (1975–1985) of HCHs and DDTs in the open ocean atmosphere and hydrosphere,' in "Long Range Transport of Pesticides," ed. D.A. Kurtz, Lewis, Chelsea, MI, pp. 127–141 (1990).
62. D.F. Rawn, T.H. Halldorson, B.D. Larson, and C.G. Muir, *J. Environ. Qual.,* **28**, 898 (1999).
63. J.N. Seiber, B.W. Wilson, and M.M. McChesney, *Environ. Sci. Technol.,* **27**, 2236 (1993).
64. EPA, Ambient Air Quality Surveillance Criteria, 40 CFR, Part 58.12 (1999).
65. California Air Resources Board, "Quality Assurance Plan for Pesticide Monitoring," California Air Resources Board, Sacramento, CA (1999).
66. J.S. Lenoir, L.L. McConnell, G.M. Fellers, T.M. Cahill, and J.N. Seiber, *Environ. Toxicol. Chem.,* **18**, 2715 (1999).
67. L.L. McConnell, E. Nelson, C.P. Rice, J.E. Baker, W.E. Johnson, J.A. Harman, and K. Bialek *Environ. Sci. Technol.,* **31**, 1390 (1997).
68. R.A. Reisinger and E. Robinson, *J. Appl. Meteorol.,* **15**, 836 (1976).
69. P.S. Honaganahalli and J.N. Seiber, *Atmos. Environ.,* **34**, 3511 (2000).
70. J.E. Woodrow, D.G. Crosby, T. Mast, K.W. Moilanen, and J.N. Seiber, *J. Agric. Food Chem.,* **26**, 1312 (1978).

Biological sampling: determining routes of wildlife exposure to pesticides

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1 Introduction

When risks to wildlife are evaluated, avian or small mammalian species are most often considered.[1–3] A separate group of regulations specify studies to evaluate aquatic organisms.[1,2,4] When evaluating wildlife, risks of adverse exposure must be quantified by determining residue concentrations of parent compounds and/or degradation products in surface soil, plant materials, food items (insects, earthworms, etc.) and/or water to document pesticide occurrence in the study area and for the study duration. There is also the possibility that reptiles or amphibians, rather than birds, may be selected as indicator species. In this case, pesticide concentrations should also be evaluated in sediments. These data provide the foundation to test hypotheses regarding exposure of nontarget species to pesticides.

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The first step in a wildlife exposure assessment is to document the occurrence and persistence of a pesticide in the study area throughout the study duration. Several articles in this book describe the experimental designs and best practices to conduct field crop and environmental dissipation (air, soil and water) studies. This article presents methods to quantify spatial and temporal distributions of pesticide presence in ecosystems following normal application and resultant exposure of nontarget wildlife.

Exposure routes for nontarget animals include ingestion of pesticide-containing food, inhalation and dermal contact. Ingestion is considered the primary route of pesticide exposure for wildlife.[3,5,6] This route is the easiest to quantify since representative food items such as plant material, insects and earthworms can be collected within the foraging areas of avian or mammalian study species. Also, food items may be collected nonlethally using standard esophageal restriction methods.[7,8] To implement successfully an assessment of pesticide ingestion, it is critical to monitor food consumption (see below) by representative species (see below) from the different phyla and genera in the study area. Study species should also be selected to represent as many feeding guilds as practical. With this wide array of potential exposure routes, data describing ingestion rates and pesticide occurrence in/on food items provide critical information for proper evaluation of chemical exposure and potential effect.

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Other exposure routes, dermal and inhalation, are less frequently evaluated since pesticide uptake by routes other than ingestion is poorly described for most species. Dermal exposure information is available from laboratory studies for many pesticides, but these data are generated using exposure scenarios that are unrealistic in the environment. Poor characterization is also due to the difficulty of presenting a dermal exposure without concomitant inhalation or ingestion. Inhalation is usually not evaluated in field trials designed to determine wildlife effects, since the data regarding inhalation are meager for wildlife species of concern,[6] and total inhalation is considered small relative to ingestion pathways.[3,5,6] Maternal transfer of crop protection products to offspring is generally considered to be negligible with most current use (nonpersistent) insecticides. As pesticides are targeted at specific biochemical pathways and optimized for specific biochemistries of pest species, more persistence may be tolerated for these highly specific pesticides. With newly developed persistent insecticides, significant maternal transfer has been observed during laboratory studies with deleterious effects on avian hatchability.[9] This route of exposure should be considered when chemical half-lives in the environment or in the body exceed the time required to conceive and rear one litter/clutch of offspring.

Plant materials contribute to dermal and ingestion routes of exposure for animals.[10–15] Foliage is often the target of pesticide application. Hence pesticide residue quantitation must be considered in plant materials (root, foliage and seeds) that are likely to be ingested by wildlife species that are considered to be at risk. In these cases, analysis of plant material may constitute a major part of verifying the spatial distribution of applied pesticide. Foliar residues often occur as dislodgeable residues from spray applications. Systemic compounds/degradation products can reach both the root and foliage in biologically relevant concentrations.[11,12] In either case, wildlife ingesting this foliage will be exposed to pesticide residues.

Chemical analyses have provided good measures of avian and mammalian exposure to pesticides.[16–27] These analyses are particularly powerful when used in comprehensive ecotoxicological evaluations,[7,20,28,29] designed to assess pesticide risks to wildlife (see Section 1.1). In such studies, representative types of organisms from the study area should be selected to serve as indicators of exposure and/or effect. Species selection criteria include both sensitivity to the test compound and the likelihood that the species will be exposed to the compound. A wide variety of indicator species can be selected based on these criteria. For example, if insecticides are being studied, insectivorous vertebrates are most likely to be considered, and other organisms would be considered at risk only if their food preferences included materials likely to experience pesticide treatment/uptake. The extent to which these indicator species are exposed to a given pesticide is readily established by analyzing food items.[8,15,16,19] These food item collections are possible in large-scale regional studies designed to evaluate risks at the population/metapopulation level.[7] Food items can be targeted based on knowledge of feeding strategies of the indicator species; for example, ingestion risks for quail may be evaluated by selection of various seed types, while assessments for robins would require attention to soil-dwelling invertebrates also. When using this approach, care should be taken to obtain feeding strategies in the ecosystems being studied and not to accept generic strategies that may poorly represent specific agroecosystem being evaluated. Using the example from above, the types of seeds preferred by quail and the types of earthworms available to robins may

be different in different agroecosystems. This problem can be overcome in studies of many avian species by sampling food items directly from indicator species.[7,8,29] This approach requires frequent visits to the nest or filming the feeding behavior of study species to guide human collection of food items. Although the former process is a direct measure, both procedures allow reliable assessment of pesticide ingestion by indicator species.

1.1 Regulatory requirements and guidelines

Within the USA, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requires that pesticide hazards be determined before registration and as part of pesticide reregistration. Registration and reregistration are contingent upon demonstrated acceptable risk from exposure to the pesticide.[1–3] Previous regulatory policies required full-scale field studies, wherein risks from a given pesticide were evaluated for multiple receptor species in all ecoregions that were known (or proposed) to receive large-scale pesticide treatment. However, current regulatory policy has significantly delayed these tests in the registration process. As part of current ecological risk assessment guidelines,[1–3] terrestrial and aquatic systems are treated differently. In assessing risks to terrestrial organisms, avian species are the primary species to be considered. Laboratory studies are largely performed at Tier 1, and limited field data such as species distributions in pertinent ecoregions, pesticide occurrence in likely food items of sensitive species and areas receiving pesticide treatment are evaluated in Tier 2.[1–3] Probabilistic evaluation of pesticide risks should be assessed in the field at Tier 3 of the assessment process. Under the new guidance, few pesticides have reached the stage that requires an assessment of their potential effects on reproduction and mortality in the field. As more products reach the market and risk assessment models suggest potential mortality in the field, assessment of wildlife exposure to pesticides and resultant effects are likely to become necessary.

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European Union regulations[30] require ongoing monitoring of potential ecological impacts if risks of mortality are high for nontarget vertebrates. Such monitoring is triggered if the worst case residue occurrence in/on wildlife food items is predicted to be 10 times the LD_{50} for a sensitive species occupying the agroecosystem in which the pesticide will be applied.[30] Scenarios that trigger such monitoring are infrequent, but do still occur. Some aspects of the studies presented below could be useful in those monitoring efforts.

1.2 Historical perspectives

Potential effects of pesticide exposure to nontarget organisms can be estimated in a number of indirect ways. In efforts to reduce costs in the regulatory process, modeling has become the most common technique for estimating effects. However, potentially large errors are the trade-off for reduced cost. In the new US Environmental Protection Agency (EPA) paradigms, few agricultural chemicals have progressed to the point within the regulatory process where field validation of potential adverse effects is required. However, this verification is part of higher tier (also termed ‘levels of

refinement' in some EPA documents) risk assessments. It is in this verification process that studies will be needed on large spatial scales. For this reason, data are presented from two case studies that were conducted when full-scale field studies were required of registrants in the USA. The design and implementation of these studies should benefit those attempting to verify modeling output of higher tier risk assessments and to provide examples illustrating the protocol design and best practices necessary to conduct field biological monitoring studies.

2 Study designs and best practices

Once exposure of indicator species is established by food item analysis, uptake is most often quantified by analysis of organ tissues.[23,24,29,31–34] For most current use pesticides, residues are not present in target tissues for extended periods, and concentrations do not increase following repeated low-dose exposures. Rather, the clearance rates for pesticides and their transformation products are relatively rapid.[35] Residues are often determined in gastrointestinal (GI) tract or liver. Analysis of blood may also provide exposure information, but detection limits are poor for most study species since blood is normally collected nonlethally. Hence smaller blood samples are collected relative to other matrices, on a dry weight basis. The advantage of blood sampling is that the technique can be performed nonlethally, allowing the indicator species to survive for further observation.[22] Excrement has also been used to estimate uptake, although material that passes through the GI tract without uptake can be present in excrement, thus incorrectly elevating the estimate of uptake by the study species. This is why studies emphasizing pesticide uptake by earthworms include a step where earthworm GI tracts are purged. One way to minimize the problem of collecting excreta that has not actually been incorporated into mammalian tissues is to evaluate urine, which by definition contains materials filtered from the blood by the kidney. Another viable approach is to quantify specific transformation products that are unlikely to be formed without exposure to the pesticide or one of its toxic metabolites.[19] The analysis of total carcass can also be performed to determine residues, but this method minimizes the concentration of important analytes by diluting them throughout the entire mass of the organism. While whole-body analyses offer the possibility of obtaining uptake data when low detection limits are not achievable, target tissues and nonlethally collected samples are preferable from study design and natural resource preservation perspectives.[7,29,36–39]

To evaluate realistic exposure scenarios properly, study sites must be selected with great care to encompass a distribution of site characteristics (see Section 2.5) while maintaining enough similarities to allow appropriate statistical comparisons. It is often advantageous to have replicated fields of different characteristics, such as edge habitat, topography, drainage patterns or soil type. The latter characteristic should be evaluated closely to avoid runoff events into small water bodies. Such runoff is unusual, but can occur during incidences of unusually high rainfall. In grain crops, hedgerow presence and previous uses are additional critical characteristics. In orchard ecosystems, investigators should consider orchard maturity, proximity to other orchards and irrigation source. It should also be noted that in some ecoareas the majority of crops must be irrigated.

2.1 *Define study objectives*

Biological monitoring studies are designed to evaluate actual exposures and effects at the highest tiers of pesticide ecological risk assessments. With this in mind, hypotheses should be stated clearly before studies are designed to allow appropriate methodologies, sample replication and quality assurance (QA) oversight in the field and the laboratory. Two critical objectives that must be addressed are the extent of effect that a study will strive to detect and the desired confidence that the effect was in fact manifested. Two case studies described in this article were designed and implemented to measure organophosphate insecticide residue occurrence in agroecosystems under normal-use scenarios and resultant wildlife exposure. Study designs specifically evaluated avian species and emphasized nestling exposures as this life stage has been found to be most sensitive to anticholinergic agents.[39] This design allows the sensitive evaluation of passerine exposures to insecticides and illustrates the objectives for other monitoring studies that may be needed for other terrestrial species.

Studies may be designed for estimating exposures to a wide array of wildlife, including birds, mammals and amphibians. Many regulatory requirements involve birds, and less emphasis is currently placed on other species. As regulatory requirements evolve, ecological risk assessments will be required for more species. This may require alternative approaches for food item analysis to allow estimates of pesticide ingestion.

One shortcoming in many field studies is a failure to address adequately exposure to toxic transformation products. In efforts to manage time and cost constraints, the concentrations of parent materials and transformation products are often added together to produce a total 'toxic residue' amount.[3,40,41] However, it is more appropriate to evaluate individual transformation products as their toxicity may be significantly increased (e.g. active oxons) or decreased (e.g. dehalogenation or dealkylation products) relative to the parent compound.[10,41–45]

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2.2 *Preparation of study protocol*

2.2.1 Role and responsibility of study personnel

When designing and implementing field studies to evaluate pesticide dissipation and ecological effect, communication between ecologists, chemists, toxicologists and often agricultural engineers is critical to a successful study. This communication must begin when protocol development begins. Sponsor representatives should relay the scope of the study and the questions to be answered. In many cases the sponsor representative is fully engaged in protocol development. The Study Director has overall responsibility for protocol development. Perhaps the most important duty of the Study Director is to organize a team of capable individuals who are committed to conducting the best possible study. If this is accomplished, the team will work together well to develop and implement accurate timelines and high data quality objectives for each phase of the study. Protocol development must include at a minimum one representative from each facet of the study. For dissipation and exposure studies, this means that representatives from field teams, laboratory teams and QA must sit together to develop reasonable protocols. Representatives should include not only the managers of laboratories or field operations, but should also involve experienced personnel

who will actually generate data. It is critical that each team understands the needs and constraints of other teams. For instance, the field personnel need to understand that certain container types minimize interferences, and laboratory personnel need to understand that for each food item sample collected a ladder has to be taken to a nest box and climbed. These discussions during protocol development allow solid study designs with achievable goals. It should be noted that if shipping personnel are not part of the technical or QA groups, their representatives should also be consulted to make sure that the critical transition of samples is accomplished. After the sponsor representative has made comments on the draft protocol, all members of the protocol development team should be involved in the finalization of the document. It is at this point that some protocols can have items added that are difficult to achieve for reasons of time or logistics.

2.2.2 Training of study personnel

When possible, all personnel should be employees of the same organization and personnel managers should be a team of experienced environmental scientists. Training of all study personnel must include sample handling, sample storage, data recording, data storage and safety. Training must be documented by managers of each facet of the investigation. Field personnel should be trained on site to allow site-specific logistics and potential hazards to be addressed. Of particular concern is timing of reentry following pesticide use.[46] Laboratory personnel should be trained in the specific laboratory used for the study, and in the event that more than one laboratory location is used for a study, all personnel should be trained by a single person to maximize data quality from the two laboratories.

2.3 Test substances

Chemical characteristics and environmental conditions will influence the design of field studies to assess distributions of occurrence and exposure.[11,12,23,29,47–49] Important chemical characteristics of the test substance include water solubility, K_{oc}, vapor pressure, degradation rate and potentially labile functional groups. These characteristics also need to be known for toxicologically important transformation products. One shortcoming in many field studies is a failure to address adequately exposure to toxic transformation products.

Typical formulated products should be used since biological monitoring studies are required when actual adverse exposures are predicted by lower tier risk assessments. There is also conflicting information regarding the influence of formulated and pure active ingredients. Thus formulated products are required in field studies to represent actual use scenarios. The formulation and carrier of all applied test substances should be well defined before study initiation and should be monitored during actual application. In the two case studies, Diazinon 50W (active ingredient: diazinon, CAS No. 333-41-5) and Fortress-5G (active ingredient: chlorethoxyfos, CAS No. 54593-83-8) are discussed. Diazinon 50W was applied as an aqueous emulsion or in superior oil, and Fortress-5G was applied as a granular formulation. Results from case studies showed that formulation components could alter the precision of the application made in agroecosystems. This is a parameter infrequently evaluated in field studies.

2.4 Test systems

For the purposes of these field studies, a test system is defined as a specific tract of land managed in part through use of pesticides. Test systems are normally limited to one crop or land use type and may include row crops, grains, fruits or golf courses. The tract of land, of course, has associated biota that are present naturally or as part of the management practices. These biota are also part of the test system and are normally described as test species or species of interest. Selection of test systems is critical to evaluate wildlife exposure scenarios in a sufficient number of sites within appropriate geographic regions.

2.5 Selection of test sites

The number of sites needed for a successful study often depends on specific site characteristics such as the following:

- weather patterns
- field size
- presence of adjacent fields
- edge characteristics
- general vegetative cover
- topography
- soil
- wildlife occurrence.

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In areas with little differences in characteristics, there may be no need for evaluation in multiple field types. However, for areas where these parameters are widely variable, sites must be replicated for each type of site. The number of sites required will increase if multiple regions must be evaluated. It should be pointed out that evaluating different edge characteristics usually plays a crucial role in study design as food resources and cover are often dependent on edge habitat. Ecosystems to be evaluated must represent areas of realistic test substance use. Each test site within an evaluated ecosystem must be characterized to finalize the field study duration and the frequency of sampling that is needed to obtain meaningful exposure data. Test site characteristics (see above) not only dictate pesticide transport and transformation[21,39,50–53] but also control the wildlife species that are available for study[28,54–62] and may limit access of investigators to areas likely to be impacted by pesticide use. Soil type, slope, vegetative cover, wildlife occupancy and climatic factors are primary factors to consider when selecting study sites. If the pesticide in question is used significantly in different climatic regions, design considerations should include evaluations in regions of major use. Habitat diversity surrounding sites and the management practices to enhance this diversity are critical criteria as they increase wildlife diversity and maximize the potential exposure of wildlife to test compounds.[54–62] Although this can only be quantified by wildlife surveys at each test site, edge habitat that provides good cover, nesting and burrowing locations is likely to provide a diverse wildlife population at the study sites. When possible, test sites should be located so that they are completely surrounded by areas treated with the pesticide being studied.[28] The border around

a test site should encompass the home range of test species inhabiting the test site. Ideally, when small mammals are being evaluated, the treated area beyond the test site should be large enough that any recruitment of study species to the test site will be from a treated area. It is not possible to cover this recruitment area for most bird species and it is often impractical for small rodents, but should be considered. Owing to abundance, home range and recruitment dynamics, small passerines and small rodents are often selected as test species.

It is common for site selection to require several months of intense evaluation. Selection criteria (see characteristics above) should be evaluated on-site, during the appropriate season, 1 year before research is to be performed. This timing also allows some time to develop partnerships with landowners, who must cooperate if research designs are to be successful. This is not a simple matter when conducting research with high-value crops such as fruits.[29]

2.6 Preparation of test sites

Issues important to site preparation include defining borders of study areas and establishing transects for monitoring wildlife presence and activity. This can be done with simple utility flags to designate the areas to be sampled and to designate which areas have been covered recently by observers. Carcass searching along these transects is also important to discover potential mortalities that might be missed in standard nest surveys.[63,64] Carcass searching includes a number of specific procedures that are likely to be site specific. Important procedures include consistent time of day, consistent search duration, new search area each day, consistent amount of area searched each day, adequate inspection of accessible edge areas and quantitation of search efficiency for each searcher. Transects or trapping grids are also the best accepted means of monitoring rodent activity on test sites.[65–68]

Specifications for nest box placement and predator guards should also be included. Specifics of nest box placement will depend on the behavior of avian species being monitored. Inter-box distances and orientation to test areas are two parameters that may vary widely with the organism being studied. Predators vary from area to area, and if nest boxes are near tree canopies, predation from above may in fact be the dominant route of predator intrusion rather than the standard access from the ground.

Since large-scale field studies most often involve accessing private property, measures for contacting landowners regarding routine and nonroutine activities is essential. Developing and maintaining good relationships with landowners is essential for long-term utilization of sufficient acreages in different ecoregions.

2.7 Application phase

2.7.1 Preparation of application media

Protocols to determine exposure scenarios should require that application be made using normal practices. Test substance application must be thoroughly documented by researchers. Documentation should include weights and volumes of materials added to

spray tanks or hoppers. Granular products should be weighed before placement in application equipment. The time of preparation and application should also be recorded.

2.7.2 Calibration of application equipment

Protocols must specify calibration of application equipment before and after application to determine the rate of product delivery when application equipment was traveling at a constant ground speed. Liquid or emulsion samples should be collected from spray nozzles and granule collection should occur as the test substance exits the application equipment. Once the correct ground speed has been determined for a given application system, that speed is maintained throughout the application process.

Protocols should require documentation of actual application practices and times. Samples of applied pesticides should be collected to document application rates to study sites. The test substance must be applied with typical equipment used for the crop, and the application must be made in accordance with the labeled use. Another variable that impacts such studies is the fact that most landowners have their own application equipment, which increases the variance in actual application rates among fields and may cause differential intra-field heterogeneity in application rates.

2.8 Sampling

Protocols must specify the number of study sites to be sampled for residues and the number of stations within a study site to be sampled. This is a difficult process to specify, but depends on traditional sampling theory.[69] In general, the parameters that need to be evaluated for proper sampling design are the following:

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- What materials are likely to contain pesticide residues following application?
- What are the known degradation kinetics of the crop protection product being evaluated?
- What are the known errors in quantifying residues in different matrices?
- What organisms are considered to be at risk, and at what life stages?
- What extent of adverse effect is acceptable?
- What confidence is desired in evaluating this effect threshold?

Protocols should also specify biotic and abiotic sample types to be collected from each sampling station and the intervals for sampling. Biotic samples should come from potentially contaminated food items (seeds, soil-dwelling invertebrates, flying insects), study species (birds, mammals) or collected carcasses. Abiotic samples normally include soil, water and less frequently sediments. Strategies and techniques for compositing both biotic and abiotic samples should also be part of the protocol to minimize the costs of performing analyses. Collection tools, storage containers and storage conditions must be specified.

Chemical occurrence and degradation must be evaluated in the matrix to which the pesticide is applied and in the matrices that are likely to receive unintentional pesticide deposition. Target areas are likely to be soil or foliage, and nontarget areas could be edge habitat or other areas that might receive significant pesticide drift. Requisite sampling should begin as near as practical to the time of application, and sampling

frequency should be based on laboratory-derived degradation rates. Significant replication within and among study fields is critical to obtain sufficient information to produce degradation rates with reasonable confidence intervals.

Since ecological parameters vary widely within and among ecoregions, the number of study sites will vary depending on the geographic range of the crop and variable cropping practices. In general, a minimum of three treatment sites are required to represent each type of site (block) within the ecoregion. In the case studies described later, four sites were selected to represent chemical degradation in each ecoregion and 10 treatment sites were monitored to evaluate potential exposure to birds from application of diazinon to apples. Of the eight treatment sites chosen for the chlorethoxyfos study, four were sampled for residue occurrence in soils and four were abandoned when adverse weather prevented application during the target time frame. More sites may be required when the test chemical is used over a wide geographic area or is used in crops that require widely different management practices or that produce significantly different habitat types within the managed area.

One fact that is often given too little attention is the actual variability inherent in pesticide application under normal use scenarios. Part of the reason for discounting this variability is that well controlled pesticide applications are often made with standard deviations that are ±40% of the mean. Using this variance, and the equation

$$N = (z\sigma/\varepsilon)^2 \tag{1}$$

where $N =$ number of samples needed to obtain a desired confidence in a given estimate, $z =$ standard normal variant (1.96 for 95% confidence), $\sigma =$ standard deviation of the observation and $\varepsilon =$ acceptable difference between actual mean and estimated mean, 16 sampling sites are needed per field if the research hopes to achieve a 95% confidence that estimates of application rates fall within 20% of the actual mean. These sampling sites need to be evaluated with sufficient frequency to establish pesticide presence in the study area and possible exposure routes for nontarget species. It should also be noted that variance will be reduced if composite samples are taken from each sampling site, and generally individual samples of biotic media are analyzed to obtain the distribution of residues needed for contemporary risk assessments.

2.9 Sample handling and shipment

Protocols normally specify that, once collected, all field samples will be immediately double-bagged, placed on dry-ice and then transported to field headquarters where they are logged in and placed in a designated freezer. Glass sampling containers may also be more appropriate to minimize interferences but normally increase shipping weights and thus costs. Freezer temperatures should be monitored daily, if not continuously. It is normally a good idea to store control and treatment samples in different coolers and in different freezers. Sample segregation should also continue for shipment to off-site facilities if required. For some chemicals, rapid dissipation may require special storage or analysis considerations. Attention to this detail can mean the difference between good quality data and uninterpretable data.

Each utensil contacting the samples should be thoroughly washed with soap that is reasonably free of components that might interfere with chemical analyses. Utensils should then be washed with water and acetone between sample collection. A separate set of collection utensils should be assigned to each field to avoid inter-field cross-contamination. Proper data recording of sampling locations within a study site and sampling area are critical to maintaining data quality. Sample placement in coolers should occur immediately after sample collection to minimize pesticide volatilization or degradation in the sample container.

2.10 QA and field data requirements

Audits of each phase of the study should include personnel training, preparation of collection forms, application calibration, each sample collection procedure, sample transport, each type of chemical analysis, data recording, data entry, data verification and data storage. Data collection in the field is often tedious if automated logging devices are not in place. To ensure data integrity, the paper and ink used for field studies should be waterproof. Each data collection form should contain appropriate locations for information detailing the time and location of sample collection, sample transport and sample analysis. Data collection forms should be stored in an orderly fashion in a secure location immediately upon return of field teams from the field at the end of each day. It is also important for data quality for studies to collect necessary field data seven days per week when required. In our experience, poor study quality is likely when field sample and data collection do not proceed on weekends.

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2.11 Data reporting

Each data point must be transferred from data sheets into spreadsheets or databases. Verification of each datum should be performed by an individual who did not enter the data being verified. Audits of each phase of the study should be performed (i.e. preparation of collection forms, application calibration, each type of sample collection, sample transport, each type of chemical analysis, data recording, data entry, data verification and data storage).

2.12 Data presentation and interpretation

Data presentations should include the parent compound and all toxic transformation products. This is particularly important for oxidation of sulfide linkages to sulfoxides or sulfones. These products are often equally toxic to the parent with increased availability. Attention should also be given to oxidative desulfuration of phosphorothionate esters.

Data should show pesticide occurrence and dissipation in important matrices during the study period (Figure 1). This has been extensively covered in other articles and will not be elaborated here. Exposure routes should be characterized well enough to quantify the dosages that are experienced by nontarget organisms. This is often

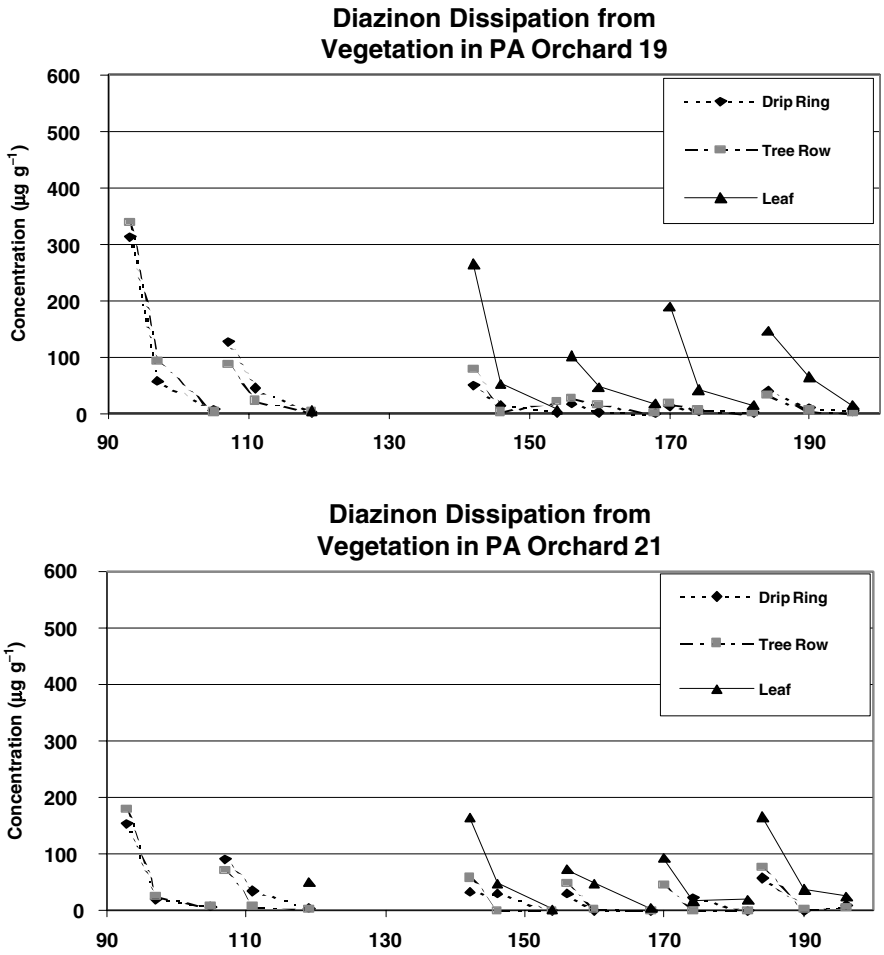


Figure 1 Mean diazinon dissipation from vegetation collected within two orchards in Pennsylvania following five applications spanning Julian days 93–187. Note that leaves were infrequently present until Julian day 120

difficult and may require knowledge of toxicokinetics for the test chemicals during laboratory studies.

The data in each table and figure of reports submitted to sponsors should be verified by QA personnel. Team leaders, laboratory managers, field managers and the Study Director should meet routinely to discuss the meaning of the data as the study develops. This allows early discussions regarding data interpretation and allows several viewpoints to be explored, which ultimately strengthen the final report for the study.

3 Case studies overview

Crop protection chemicals undergoing field testing to determine dissipation, wildlife exposure and toxicological effects will have undergone extensive laboratory tests to

evaluate their potential to cause adverse effects in wildlife.[1–3] Chemical characteristics, mode of action and area of pesticide application are critical factors in determining the probability of adverse ecological effects. If laboratory data indicate the potential for unacceptable risk, exposure assessment data are needed for nontarget organisms that inhabit ecosystems in which the pesticide is most likely to be used. In current risk assessment processes, distributions of chemical occurrence, persistence and effect are essential to develop probabilities of adverse effect.[1–3,70,71] Distributions of chemical occurrence and the longevity of effects are poorly defined in agroecosystems. These parameters are necessary to reduce the uncertainty in risk assessment processes. Adverse effects predicted by these risk assessments should be validated in well designed field studies.

Numerous field studies have been designed and conducted to evaluate pesticide impacts on wildlife. Some of these are published, and many others were submitted to support chemical registration or reregistration.[20,28,29,72–74] The latter group of studies is more difficult to access due to EPA sensitivity to confidentiality. The remainder of this article will address some of the design and implementation considerations for reregistration studies involving Diazinon 50W and for registration studies involving Fortress-5G. Overview findings documenting observed distributions for insecticide application, dissipation and uptake will be emphasized.

3.1 Case study with Diazinon 50W

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Diazinon is a widely used agricultural and residential insecticide.[75] Biological monitoring studies for diazinon in orchard ecosystems were located in south central Pennsylvania (PA), and in central Washington (WA). Habitat diversity, isolation from other orchards and orchardist cooperation were primary considerations for the study.[29] Habitat diversity was of concern to maximize the number of potential species inhabiting study sites, and orchard isolation was essential to minimize exposure of study species to crop protection products from other cultivated areas. When evaluating ecological effects in fruit orchards, the need to control pest management practices also allows the possibility of severe crop damage. Finding orchardists who agree to control their management practices is time consuming and expensive. Of 20 study sites in each State, four treatment sites and one control site were randomly selected in each State and sampled for residues.[29]

3.1.1 Methods

Diazinon 50W was applied by air blast sprayers in accordance with typical application practices for orchards. Application began in March and continued until early- to mid-July. Dormant sprays typically contained diazinon in an oil mixture.[29] Aqueous emulsions were applied as foliar sprays thereafter. Equipment was calibrated to provide an application rate of 3.4 kg active ingredient (a.i.) ha^{-1}.[18,29] At least five applications were made at approximately 2-week intervals. During these applications, 233 samples were taken from spray tanks across the four treatment fields to estimate the application rate in PA, and 244 samples were collected in WA.

All field procedures have been described in detail[18,29] and the following samples were obtained. In each of eight orchards, apple leaves (LV), under story vegetation beneath the tree canopy (DR) and under story vegetation within tree rows (TR), were collected in predetermined locations with shears. Earthworms were collected by digging to 25-cm depth in approximately a 1-m^2 area. Environmental samples were collected from each station before the first diazinon application and additional sampling occurred at 0, 4 and 12 days post-application (D0, D4 and D12). Pesticide ingestion by avian nestlings was quantified using esophageal restriction.[8,29] Diazinon was also determined in avian GI tracts of juvenile nestlings collected at 15 days post-hatch or carcasses found during daily searches.[29]

Each vegetation, soil or tissue sample was uniquely numbered and stored individually in a plastic Ziploc bag.[29] Samples were frozen until shipped to laboratory facilities. Samples were shipped in coolers with dry-ice and were returned to freezers immediately upon receipt at the analytical laboratories. Control samples were stored separately from treated samples.

(1) Chemical analysis. Tissues were homogenized before extraction. Diazinon and diazoxon was recovered from samples with *n*-hexane–acetone solvent extraction. Each sample was fortified with chlorpyrifos, as a reference standard, to determine the recovery during each extraction. Three portions of solvent were used, and the combined extract for each sample was dried with sodium sulfate. Analyses employed gas chromatography/flame photometric detection. Limits of detection for vegetation and animal tissues were 0.2 and 0.007 $\mu g\, g^{-1}$, respectively. Recoveries from fortified samples were 82%.[29] Diazoxon occurrence was infrequent and at trace concentrations. Therefore, the data presented and discussed below address only diazinon.

3.1.2 Critical observations

(1) Distributions of residues are needed to estimate pesticide exposure. Spatial heterogeneity of diazinon residues demonstrated that exposure distributions are needed to estimate hazards to nontarget organisms (Table 1). The range of residue concentrations may be caused by variability in application rates, dissipation rates and by interception of the food item by the applied spray. Residue analyses of tank samples showed that the average application rates were within 10% of the nominal application rates. The average rate was 3.16 ± 0.20 kg a.i. ha^{-1} (mean $\pm$ SE) in PA and 3.06 ± 0.14 kg a.i. ha^{-1} in WA, although the measured application rates varied among orchards. The more important information available from the many analyses of spray tank samples was the distribution of insecticide applied across a given field on a given day. These distributions contained many values near the mean application rate, but a few spray tank samples were removed from the mean by a factor of six. In this and other distributions discussed, the general shape of application distributions can be described by skewed distributions such as logarithmic or beta. Considering all foliar and dormant sprays, 74% of applications in PA and 72% in WA were within the range 2–4 kg a.i. ha^{-1}. When evaluating foliar applications only, this range was achieved in 77 and 91% of cases from PA and WA, respectively. The difference in the application precision demonstrates that dormant spray (oil-based) applications were

Table 1 Diazinon concentrations in crop samples collected from European starlings (*Sturnus vulgarus*) following several applications to orchards in eastern Washington, USA

				Residues[a]		
				Geometric mean	Confidence limit (95%)[d]	
Application	DPA[b]	*N*	*n*[c]		Low	High
1	27	1	1	0.173		
2	0	1	0	<0.007		
	1	2	1	0.013	0.004	0.045
	2	1	0	<0.007		
	3	4	2	0.035	0.005	0.250
	4	3	1	0.020	0.003	0.135
	5	6	3	0.012	0.004	0.033
	6	9	0	<0.007		
	7	7	3	0.020	0.003	0.143
	8	5	0	<0.007		
	9	3	0	<0.007		
	10	11	3	0.015	0.002	0.114
	12	4	3	0.057	0.011	0.292
3	0	17	9	0.013	0.003	0.059
	1	3	1	0.010	0.004	0.027
	3	5	0	<0.007		
	5	1	1	0.015		
	10	1	1	0.006		
4	8	2	0	<0.007		
	12	2	0	<0.007		

[a] Residues of diazinon are reported in $\mu g\ g^{-1}$.
[b] DPA = days post-application.
[c] n = number of sample types with detectable residues from a total sample size N.
[d] Confidence limits can only be calculated if $N > 1$ and $n > 0$.

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more variable than were foliar sprays (aqueous emulsions). The range of measured application rates demonstrates the need for large sample numbers in order to obtain realistic estimates of spatial heterogeneity in pesticide concentrations.

Dissipation of dislodgeable residues from vegetation displays significant spatial and temporal heterogeneity, as shown in Figure 1. Pesticide presence is a critical factor in exposure scenarios, and different degradation rates will heavily influence the potential for wildlife to become exposed to pesticides. Simple exponential functions describe 87–94% of the variance in diazinon dissipation[29] and predict that leaves should retain 6% of applied diazinon until D14, while 2% of diazinon should remain on under story vegetation at D14. The interfield distribution of degradation rates observed for each vegetation type in each field shows pseudo-first-order degradation rate constants (k) that vary from 0.04 to 0.56 day^{-1} with the majority of rate constants falling within 0.02 day^{-1} of the mean. The measured diazinon degradation demonstrated different dissipation across ecoregions and provides a useful basis for parameterization of exposure assessments within probabilistic risk assessments.[1–4]

(2) Large numbers of samples are required to characterize exposure distributions. Starling food items contained the highest diazinon concentrations and the highest

frequency of diazinon detection during the first 5 days following application. Of 88 samples taken during the study, 29 contained detectable residues, of which 18 samples were obtained within 5 days of application (Table 1). Diazinon presence in food items is also a function of the site from which the food item was obtained. For example, in WA orchard sites, the majority of food item samples containing detectable diazinon concentrations were collected from 3 of 10 study sites. Also in that region, the most frequent earthworm exposure across all sites occurred following the second and third applications, when most avian nests contained chicks. Since the diazinon concentrations were highly variable, characterizing the exposure of birds to diazinon required an extensive sampling regime at both geographic locations.

Pesticide concentrations in earthworms displayed regional differences. Such differences are likely to be observed in other potential food items. Earthworm exposure was log-normally distributed with the highest concentration being 163 $\mu g\,g^{-1}$, which represents the 97th percentile of diazinon found in earthworms from all sites.[29] The geometric mean diazinon concentration in earthworms from PA was 2.56 $\mu g\,g^{-1}$ ($CL_{95} = 1.62$–$4.06\ \mu g\,g^{-1}$), and from WA was 0.046 $\mu g\,g^{-1}$ ($CL_{95} = 0.008$–$0.28\ \mu g\,g^{-1}$). Diazinon concentrations in earthworm samples were higher ($p < 0.005$) in PA orchards, where rainfall was frequent, than in the more arid WA orchards. This difference also existed for live captured earthworms from PA and WA ($p < 0.017$). A large number of samples are required to detect differences in pesticide exposures from living and dead invertebrates with confidence. Vertebrate exposures can be influenced by differential residue concentration for living and dead/moribund food items.

(3) Avian ingestion of pesticides by avian species varies temporally, spatially and among study species. Avian species ingesting diazinon were markedly different in the two ecoregions studied (Table 2). European starlings (the species attracted to the sites) and American robins were the species that inhabited sites in both ecoregions with regularity. Even though diazinon was present in GI tracts from 96% of avian species across treated fields in both ecoregions,[28] exposure distributions were log-normal, as indicated by means and confidence limits in Table 2. Interestingly, higher exposures occurred within 12 days post-application (DPA). Owing to the high frequency of low exposures, mean exposures computed from samples collected within 12 DPA did not significantly alter geometric mean concentrations compared with means of all exposures for the key species presented. Intensive sampling at early time points was essential for characterization of exposures.

Diazinon concentrations found in live earthworms highlight the potential for secondary exposure of verminivores in orchard ecosystems. Earthworms are common items in the diet of many birds and are consumed by various other vertebrates.[16,40] Given the diazinon concentrations found in earthworms and the relative number of earthworms available for consumption in the two areas, the risk of birds ingesting organophosphate-containing earthworms is significantly greater in the PA than in the WA orchards. Diazinon uptake by earthworms in WA was similar to that found in other studies,[16,44] but concentrations found in earthworms from PA were much higher, highlighting the differences in exposure that may be observed in different geographic regions.

Table 2 Diazinon concentrations[a] in gastrointestinal tracts[b] of avian species inhabiting apple orchards following multiple treatments

		Days 0–12 post-application		
Location	Species	*N*	*n*[c]	Diazinon concentration[d]
WA	American robin	18	12	0.031 (0.004–0.233)
	Brown-headed cowbird	4	2	0.006 (0.002–0.022)
	Canada goose	12	12	1.622 (0.656–4.011)
	European starling	132	34	0.007 (0.003–0.016)
	House finch	4	2	0.009 (0.004–0.020)
	Killdeer	4	4	1.746 (1.319–2.313)
	Western meadowlark	4	2	0.022 (0.003–0.139)
PA	American robin	17	15	0.044 (0.008–0.239)
	Brown-headed cowbird	7	3	0.009 (0.003–0.025)
	European starling	72	12	0.011 (0.002–0.052)
	Mourning dove	7	4	0.018 (0.006–0.053)
	Northern cardinal	18	9	0.009 (0.004–0.018)

[a] Residues of diazinon are reported in $\mu g\, g^{-1}$.
[b] Data are presented for species represented by four or more individuals.
[c] Number of samples with detectable diazinon concentrations.
[d] Geometric mean and (in parentheses) 95% confidence limit.

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3.2 Fortress-5G case study

These data are presented to demonstrate some of the rigors needed to conduct field studies with crop protection products that have very short half-lives. This is an emerging issue with new generations of crop protection products. Dissipation and exposure studies were conducted for Fortress-5G, used to control corn rootworm. The active ingredient of this formulation is chlorethoxyphos (CEF), which exhibits a short half-life in the environment. Previous studies have measured the average field half-life of CEF at less than 3 days.[76] Thus chemical sampling and analysis required careful timing to minimize analyte degradation between sample collection and analysis. Rapid analytical methods were also essential to avoid analyte losses during actual processing/analysis intervals. Intervals between sample collection and analysis were also kept as constant as possible to normalize any degradation that might occur across all samples.

3.2.1 Methods

(1) Study site characterization. Research sites were selected in Iowa. Eight sites were designated for treatment with Fortress-5G, and eight sites served as controls. All sites were bordered by or contained one or more creeks. Soils were a silty loam composition, but some had a surface layer of clay loam. All sites had grassy areas, hedgerows and/or blocks of timber adjacent to or within the planted portion of the field. The number of planted acres per site ranged from 39 to 75. Four sites were lost from the study owing to wet weather conditions that prevented planting during the target time frame.

(2) Chemical application. Chemical application of Fortress-5G was made using T-band application at the maximum rate of 6 oz of Fortress-5G per 1000 ft of row. Planter chemical hoppers were calibrated and set before chemical application and checked by field personnel after the field was planted.

(3) Soil and earthworm sampling. Six sampling stations were defined for each field using stratified random sampling. Soil samples were collected as blocks (7.6-cm deep, 2.5-cm wide and 23-cm long) centered on or between furrows. Earthworms were collected by digging four trenches, 25-cm deep and 45-cm wide, along transects extending down-furrow from each of the six sampling stations. Worms from each sampling unit were washed with distilled water before collection.

Pre-treatment soils and earthworms were sampled once from control and treated sites. Four soil samples from each station were collected and pooled in the field. Each of four trenches within a station was dug until two worms were found or 3 m of soil per trench had been examined. Worms from each station were composited.

Post-treatment soil and earthworm samples were collected from all sites on Day 0 (application day), 1, 2, 3, 4, 6, 8, 12, 16 and 24 on treated sites. Samples on Day 0 were collected within 3 h of chemical application. Soil from control sites was sampled on Day 7 post-application only. One in-furrow soil sample from each control station was collected and the six samples were pooled. Within a given field, earthworms from each of the six stations were composited for analysis by combining all in-furrow samples into one composite and all between-furrow samples into another. Earthworms from control sites were sampled in-furrow on Day 8 and 16 post-planting only.

(4) Starling nest box monitoring. Forty starling nest boxes were placed 16–18 m apart around the perimeter of each study site. Boxes not occupied on Day 0 were closed. Any species other than the European starling found inhabiting a nest box was removed. The large number of wild, similarly aged starling chicks allowed exposure assessment to be determined via regularly scheduled collection of invertebrate food items and nestling mortalities. Food items were collected from the esophagus of nestlings as described previously.[8] Food items were collected twice daily (morning and afternoon) from nestlings of age 3, 9, 12 and 15 days. Any birds found dead in nest boxes were collected.

(5) Sample handling and shipment. Once collected, all samples were immediately double-bagged, placed on dry-ice and then transported to field headquarters where

they were logged in and placed in a designated freezer. Freezer temperatures were monitored daily. Owing to the rapid dissipation of CEF, each composite soil sample was mixed and subsampled at the field station. Each frozen sample was ground in a Hobart food chopper with dry-ice, then mixed three times through a soil riffler. Approximately 500 cm^3 of the mixed soil were then collected, placed in double cloth bags and returned to the freezer. All equipment and tools were cleaned with detergent and water and rinsed with acetone between each sample. Samples were shipped frozen overnight to analytical laboratories for CEF determination.

(6) CEF quantitation

(a) Sample extraction. All analytical procedures were validated, and mean recoveries for fortified samples ($n > 55$) were between 91 and 96% for all sample matrices with standard deviations <20%. Samples were extracted with pentane–diethyl ether (3:1 v/v). Solvent volume was reduced using Kuderna–Danish concentrators. Extracts from GI tracts required further cleanup with a Diol solid-phase extraction cartridge before final volume adjustment. The limit of quantitation (LOQ) was the sample extract concentration equivalent to that of the lowest analytical standard (0.01 ng μL^{-1}).

(b) Instrumental analysis. Sample extracts were analyzed for CEF using a Hewlett-Packard model 5890A gas chromatograph with flame photometric detection (P-mode). A DB-1701 megabore column, 15 m × 0.53-mm i.d., was used for chromatographic separation. Each sequence of analyses began and ended with a blank and five calibration standards, ranging in concentration from 0.010 to 2.0 ng μL^{-1}.

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3.2.2 Critical observations

(1) Spatially heterogenous distributions of crop protection products, within and among test sites, will result from well controlled applications. The soil CEF concentrations ranged from a high of 2.3 $\mu g\, g^{-1}$ at one station on site 1 Day 0 to none detected in 10 samples from various stations several days post-application. The measured mean soil CEF concentrations were between 0.2 and 1.0 $\mu g\, g^{-1}$ on Day 0. The theoretical CEF concentration immediately after application was calculated to be 1.2 $\mu g\, g^{-1}$, based on a target application rate of 6 oz of Fortress-5G per 1000 ft of row, and using 1.36 $g\, cm^{-3}$ as the average soil density.[77] These data further demonstrate the substantial distribution of measured application rates within a highly controlled study, and indicate a need for more rapid analysis of samples after collection, perhaps in field laboratories.

(2) Distributions of pesticide concentrations in potential food items for avian species are required to estimate the contribution of food to exposure of birds in different regions where the test chemical may be used. On treated fields, detectable CEF residues were found in 102 of 207 earthworm samples. No earthworm samples collected from control fields ($N = 28$) contained detectable CEF. Average CEF concentrations in earthworms reached maxima 1–4 days post-application (Table 3). Mean CEF residues in earthworms fell below 0.1 $\mu g\, g^{-1}$ after 8 days post-application. This

Table 3 Chlorethoxyfos concentrations[a] found in earthworms collected from corn agroecosystems in south Central Iowa

DPA[b]	N[c]	n[c]	Mean	SD	Max.	Min.[d]
1	31	9	0.10	0.29	1.5	<0.015
2	31	13	0.11	0.24	1.2	<0.015
3	28	17	0.12	0.19	0.88	<0.015
4	28	13	0.15	0.30	1.3	<0.015
6	27	12	0.099	0.16	0.67	<0.015
8	25	18	0.082	0.11	0.35	<0.015
12	10	8	0.072	0.063	0.21	<0.015
16	17	9	0.081	0.13	0.45	<0.015
24	10	3	0.025	0.040	0.14	<0.015

[a] Residues are reported in $\mu g\, g^{-1}$.
[b] Days post-application.
[c] n = Number of samples with detectable concentrations from a total of N samples.
[d] These values represent detection limits.

is a relatively quick dissipation of residues and is an important factor in assessing the potential exposure duration in worm-eating birds.

Of those matrices measured, worms collected from treated fields present the most significant potential route of exposure for those species of wildlife which rely on them as a major food source. Worms were the matrix, aside from soil, with the highest frequency of detection (49% of samples collected on treated sites post-application) and the highest mean concentrations (0.025–0.15 $\mu g\, g^{-1}$ on treated sites).

Invertebrate food items, collected from starling nestlings, with quantifiable residues were collected 1–5 days post-application. Sixteen of the 178 (9.2%) samples contained detectable CEF residues at concentrations ranging from 0.013 to 2.6 $\mu g\, g^{-1}$. Daily averages, including detectable and nondetectable residues, ranged from 0.009 to 0.017 $\mu g\, g^{-1}$. *Lepidoptera* larvae made up 84% of the biomass in nestling diets, and 14% of the samples containing CEF residues were in this insect order. Order *Coleoptera* comprised 3% of the biomass in the nestlings' diet, but represented 82% of the samples in which CEF residues were found. Several other orders that were present but represented less than 4% of the total biomass included *Diptera, Glomerida, Hemiptera, Homoptera, Isopoda, Neuroptera, Orthoptera*, and *Phalangida*. This distribution of pesticide occurrence in specific insect orders illustrates the potential for pesticide exposure to change seasonally and annually depending on the abundance of certain food items.[78–80]

(3) Avian ingestion of pesticides by avian species varies spatially and temporally within and among study sites. Most pre- and post-application carcasses ($N > 130$) contained less than the CEF limit of quantitation. Six of the 94 starling carcasses collected post-planting from treated sites contained detectable CEF. The observed concentrations ranged from 0.01 to 0.26 $\mu g\, g^{-1}$, with a geometric mean of 0.022 $\mu g\, g^{-1}$ ($CL_{95} = 0.0076$–0.062). Two pairs of sibling nestlings were collected on two sites, and therefore only four nest boxes (5%) contained nestlings that had measurable CEF residue. Of those six carcasses that contained CEF residues, four were collected within 4 days of application. The two exceptions were collected 14 days post-application.

CEF dissipation profiles in soil and accumulation in biota suggest that CEF was available at significant levels for a short time relative to other organophosphates.

4 Conclusions and recommendations

Field studies represent intense efforts to quantify pesticide occurrence, dissipation, wildlife exposure and/or risk in actual field-use scenarios. The study designs presented were cutting edge at the time of implementation and present a wealth of critical data regarding pesticide occurrence and wildlife exposure in agroecosystems. The information presented in this article demonstrates the variance of pesticide distribution found within and among agroecosystems undergoing intensive pest management. Pesticide dissipation follows relatively well-known rate equations, although the specific equation may differ among pesticides. In both case studies 80% of documented pesticide ingestion by birds occurred within 4 days of application. These data are consistent with soil dissipation data and with pesticide occurrence in invertebrates and specific food items of avian species.

As with any study designs, there are always improvements that could be made. In orchard studies, some measure of leaf surface area would have improved our assessment of pesticide application to the tree canopy. The timing of application could also have been more synchronous but, given the value associated with an apple crop, orchardists were allowed a great deal of latitude in the timing of initial applications. Such considerations are inevitable in large scale field studies. During the Fortress-5G corn studies, more rapid stabilization and analysis of the samples would have improved data quality. This is a primary consideration for studies with pesticides that have rapid dissipation rates. Both studies would have benefited from electronic data logging procedures that are currently available. To encompass exposure scenarios in different crop types, focal species may change. These changes may require monitoring of natural nests, which is more difficult to accomplish.

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Temporal patterns of pesticide occurrence in agroecosystems provide critical baseline information for Tier 2 probablistic assessments of wildlife exposure to insecticides. These data are largely unavailable for current probablistic assessments, thus forcing regulators to include safety factors that increase risk estimates and prolong the registration process. Data from field studies such as those described in the case studies could provide information that will fill current data gaps and will reduce uncertainties in probabilistic risk assessments. Distributions of application rates and dissipation rate data for residues on food items are particularly useful in risk assessments. Collection of data describing exposure distributions is highly recommended to reduce uncertainties in current risk assessment processes. The logical first step in this process would be to compile existing exposure data from past field studies. This will take significant cooperation within the regulated community.

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References

1. USEPA. A Case Study: Advancing Ecological Risk Assessment Methods in the EPA. Office of Pesticide Programs, United States Environmental Protection Agency, Washington, DC (2001). Also available on the World Wide Web: **http://www.epa.gov/oscpmont/sap/2001/index.htm#march**.
2. USEPA. Implementing Probabilistic Ecological Assessments: a Consultation Insect Repellent Product Performance Testing Guideline Evaluation. Office of Pesticide Programs, United States Environmental Protection Agency, Washington, DC (2001). Also available on the World Wide Web: **http://www.epa.gov/oscpmont/sap/2000/index.htm#april**.
3. ECOFRAM: Terestrial Report. United States Environmental Protection Agency, Environmental Fate and Effects Division, Washington, DC (1999).
4. ECOFRAM: Aquatic Report. United States Environmental Protection Agency, Environmental Fate and Effects Division, Washington, DC (1999).
5. C.J. Warwick, J.D. Mumford, and G.A. Norton, *Comput. Electron. Agric.,* **7**, 47 (1992).
6. C.J. Driver, M.W. Ligotke, P. Van Voris, B.D. McVeety, B.J. Greenspan, and D.B. Drown, *Environ. Toxicol. Chem.,* **10**, 21 (1991).
7. G.P. Cobb and M.J. Hooper, 'Nonlethal wildlife monitoring to determine exposure to xenobiotics and resulting impacts,' in "The Population Ecology and Wildlife Toxicology of Agricultural Pesticide Use," eds. R.J. Kendall and T.E. Lacher, Lewis Publishers, Chelsea, MI, pp. 35–46 (1994).
8. R.S. Mellott and P.E. Woods, *J. Field Ornithol.,* **64**, 205 (1993).
9. USEPA. Review of American Cyanamid Company's Probablistic Assessment of Chlorfenapyr and Request for Guidance on Problem Formulation. In FIFRA Science Advisory Panel Report, SAP99-05. US Environmental Protection Agency, Washington, DC (1999). Also available on the World Wide Web: **http://www.epa.gov/scipoly/sap/1999/september/finalrpt.pdf**.
10. A.G. Wildeman and R.N. Nazar, *Can. J. Genet. Cytol.,* **24**, 437 (1982).
11. T.G. Pfleeger, A. Fong, R. Hayes, H. Ratsch, and C. Wickliff, *Environ. Toxicol. Chem.,* **15**, 535 (1996).
12. J.S. Fletcher, J.E. Nellessen, and T.G. Pfleeger, *Environ. Toxicol. Chem.,* **13**, 1383 (1994).
13. G.F. Antonious, M.E. Byers, and J.C. Snyder, *Pestic. Sci.,* **54**, 61 (1998).
14. P.Y. Hamey and C.A. Harris, *Regul. Toxicol. Pharmacol.,* **30**, S34 (1999).
15. T.C. Marshall and H.W. Dorough, *J. Agric. Food Chem.,* **25**, 1003 (1977).
16. G.L. Stephenson, C.D. Wren, I.C.J. Middelraad, and J.E. Warner, *Soil Biol. Biochem.,* **29**, 717 (1997).
17. J.A. Buck, L.W. Brewer, M.J. Hooper, G.P. Cobb, and R.J. Kendall, *J. Wildl. Manage.,* **60**, 321 (1996).
18. G.P. Cobb, L.W. Brewer, E.H.H. Hol, and C.M. Bens, 'Diazinon dissipation from vegetation in apple orchards from Pennsylvania and Washington,' in "ACS Symposium Series: Field Dissipation of Agrochemicals," ed. E. Authur, American Chemical Society, Washington, DC. In press.
19. M.N. Egyed, M. Makinson, A. Eilat, and A. Shlosberg, *Refuch. Veterin.,* **31**, 22 (1974).
20. L.W. Brewer and R.A. Hummell, 'What is measurable in wildlife toxicology,' in "Wildlife Toxicology and Population Modeling," eds. R.J. Kendall and T.E. Lacher, Lewis Publishers, Boca Raton, FL, pp. 69–76 (1994).

21. J.N. Seiber, B.W. Wilson, and M.M. McChesney, *Environ. Sci. Technol.,* **27**, 2236 (1993).
22. S.A. Volz, J.J. Johnston, and D.L. Griffin, *J. Agric. Food Chem.,* **49**, 2741 (2001).
23. H.N. Nigg and J.H. Stamper, *ACS Symp. Ser.,* **382**, 6 (1989).
24. J. Kammerbauer and J. Moncada, *Environ. Pollut.,* **103**, 171 (1998).
25. J.K. Kairu, *Int. J. Salt Lake Res.,* **3**, 31 (1994).
26. A. Navas Diaz, A. Garcia Pareja, and F. Garcia Sanchez, *Pestic. Sci.,* **49**, 56 (1997).
27. S.C. Ogilvie, R.J. Pierce, G.R.G. Wright, L.H. Booth, and C.T. Eason, *J. Ecol.,* **21**, 195 (1997).
28. S.L. Tank, L.W. Brewer, M.J. Hooper, and G.P. Cobb, *Environ. Toxicol. Chem.,* **12**, 2113 (1992).
29. G.P. Cobb, C.M. Bens, R.J. Kendall, R.J. Mellott, and L.W. Brewer, *Environ. Toxicol. Chem.,* **19**, 1360 (2000).
30. Directive 91/414/EEC. Annex II, §8 and Annex III §10. European Commission, Brussels (2000).
31. J.J.M. van de Sandt, W.J.A. Meuling, G.R. Elliott, N.H.P. Cnubben, and B.C. Hakkert, *Toxicol. Sci.,* **58**, 15 (2000).
32. E.R. Richardson and J.N. Seiber, *J. Agric. Food Chem.,* **41**, 416 (1993).
33. D.P. Schultz and E.W. Whitney, *Pestic. Monit. J.,* **7**, 146 (1974).
34. G.D. Johnson, D.A. Palmer, H.O. Krueger, and D.L. Fischer, *Environ. Toxicol. Chem.,* **12**, 673 (1993).
35. R.J. Kendall and J. Akerman, *Environ. Toxicol. Chem.,* **11**, 1729 (1992).
36. C.P. Weisskopf and J.N. Seiber, 'New approaches to the analysis of organophosphate metabolites in the urine of field workers,' in "ACS Symposium Series: Biological Monitoring for Pesticide Exposure: Measurement, Estimation, and Risk Reduction," eds. R.G.M. Wang, C.A. Franklin, R.C. Honeycutt, and J.C. Reinert, American Chemical Society, Washington, DC, pp. 206–214 (1989).
37. T. Bargar, W. Rhodes, C. McMurry, R.L. Dickerson, and G.P. Cobb, *Arch. Environ. Contam. Toxicol.,* **37**, 364 (1999).
38. M.C. Fossi, *Environ. Health Perspect.,* **102**, 49 (1994).
39. M.J. Hooper, L.W. Brewer, G.P. Cobb, and R.J. Kendall, 'An integrated laboratory and field approach for assessing hazards of pesticide exposure to wildlife,' in "Pesticide Effects on Terrestrial Wildlife," eds. L. Somerville and C.H. Walker, Taylor & Francis, Philadelphia, PA, pp. 271–283 (1990).
40. G.P. Cobb, E.H. Hol, P.W. Allen, J.A. Gagne, and R.J. Kendall, *Environ. Toxicol. Chem.,* **14**, 279 (1995).
41. C. Albelda, Y. Pico, J.C. Molto, G. Font, and J. Manes, *Int. J. Environ. Anal. Chem.,* **58**, 315 (1995).
42. C.H. Walker and G.O. Johnston, *Biochem Soc. Trans.,* **21**, 1066 (1993).
43. A.C. Belfroid and M. Van Drunen, *Sci. Total Environ.,* **222**, 167 (1998).
44. B.L. Roberts and H.W. Dorough, *Environ. Toxicol. Chem.,* **3**, 67 (1984).
45. K.A. Klaassen, 'Casarett and Doull's Toxicology,' sixth edition, McGraw-Hill, New York (2001).
46. W.J. Popendorf, *Am. J. Ind. Med.,* **18**, 313 (1990).
47. M.Z. Peery, *Auk,* **117**, 511 (2000).
48. G.T. Brooks, *J. Environ. Sci. Health B,* **15**, 755 (1980).
49. F.A. Elhag, W.N. Yule, and W.D. Marshall, *Bull. Environ. Contam. Toxicol.,* **42**, 172 (1989).
50. M.E. Balmer and K.U. Goss, *Environ. Sci. Technol.,* **34**, 1240 (2000).
51. C.M. Aelion and D.C. Cresci, *J. Environ. Qual.,* **28**, 683 (1999).
52. S. Susarla and M.M. El. Hefnawy, *Toxicol. Environ. Chem.,* **62**, 149 (1997).
53. P. Nkedi-Kizza and K.D. Brown, *J. Environ. Qual.,* **27**, 1318 (1998).
54. J.M. Smolen and A.T. Stone, *Soil Sci. Soc. Am. J.,* **62**, 636 (1998).
55. C. Boutin, K.E. Freemark, and D.A. Kirk, *Agric. Ecosyst. Environ.,* **72**, 239 (1999).
56. N.L. Rodenhouse and L.B. Best, *Am. Midl. Nat.,* **131**, 96 (1994).
57. D.A. Kelt and D.K. Van Vuren, *Am. Nat.,* **157**, 637 (2001).
58. P. Drapeau, A. Leduc, J.-F. Giroux, J.-P. L. Savard, Y. Bergeron, and W.L. Vickery, *Ecol. Monogr.,* **70**, 423 (2000).
59. K.A. Hobson and E. Bayne, *Wilson Bull.,* **112**, 373 (2000).
60. C. Boutin, K.E. Freemark, and D.A. Kirk, *Agric. Ecosyst. Environ.,* **72**, 239 (1999).
61. M.E. Weale, D. Whitwell, H.E. Raison, D.L. Raymond, and J.A. Allen, *Oecologia (Berlin),* **124**, 391 (2000).

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62. I.R. Inglis, E. Wright, and J. Lill, *Agric. Ecosyst. Environ.,* **48**, 257 (1994).
63. R.J. Kendall and L.W. Brewer, *J. Wildl. Dis.,* **29**, 458 (1993).
64. H.J. Homan and G. Linz, *Wildl. Soc. Bull.,* **29**, 292 (2001).
65. A. Utrera, G. Duno, B.A. Ellis, R.A. Salas, N. de Manzione, C.F. Fulhorst, R.B. Tesh, and J.N. Mills, *J. Mammal.,* **81**, 536 (2000).
66. E.E. Jorgensen and S. Demarais, *J. Mammal.,* **80**, 421 (1999).
67. C.A. Parmenter, T.L. Yates, R.R. Parmenter, J.N. Mills, J.E. Childs, M.L. Campbell, J.L. Dunnum, and J. Milner, *J. Wildl. Dis.,* **34**, 1 (1998).
68. J.M. Fryxell, J.B. Falls, E.A. Falls, and R.J. Brooks, *Ecology,* **79**, 213 (1998).
69. L.H. Keith, G.L. Patton, D.L. Lewis, and P.G. Edwards, 'Determining what kinds of samples and how many samples to analyze,' in "Principles of Sampling," ed. L.H. Keith, American Chemical Society, Washington, DC, pp. 3–40 (1996).
70. T.R. Stafford and L.B. Best, *Environ. Toxicol. Chem.,* **18**, 722 (1999).
71. C.B. Cleveland, M.A. Mayes, and S.A. Cryer, *Pest Manage. Sci.,* **58**, 7 (2002).
72. E.W. Schafer, W.A.J. Bowles, and J. Hurlbut, *Arch. Environ. Contam. Toxicol.,* **12**, 355 (1983).
73. P. Mineau, D.C. Boersma, and B. Collins, *Ecotoxicol. Environ. Saf.,* **29**, 304 (1994).
74. R. Balcomb, R. Stevens, and C. Bowell, *Bull. Environ. Contam. Toxicol.,* **44**, 302 (1984).
75. R. Eisler, Diazinon Hazards to Fish, Wildlife, and Invertebrates: a Synoptic Review, NTIS Order No. PB86-235074/GAR, US Fish and Wildlife Service, Washington, DC (1986).
76. R.V. Slates and C.D. Crowe, Field Soil Dissipation of Fortress® Soil Insecticide. Report No. AMR-1143-90. Agricultural Products Department, E.I. du Pont de Nemours, Wilmington, DE (1990).
77. G.R. Blake and K.H. Hartge, 'Bulk density,' in "Methods of Soil Analysis Part 1: Physical and Mineralogical Methods," ed. A. Klute, Second edition, American Society of Agronomy and Soil Science Society of America, Madison, WI, pp. 363–375 (1986).
78. M.A.A. van der Meij and R.G. Bout, *Can. J. Zool.,* **78**, 1668 (2000).
79. M.E. Weale, D. Whitwell, H.E. Raison, D.L. Raymond, and J.A. Allen, *Oecologia (Berlin),* **124**, 391 (2000).
80. P.J. McCarthy and D.W. Winkler, *Condor,* **101**, 246 (1999).

Best practices in conducting dislodgeable foliar residue studies

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1 Introduction

The potential health risk of workers exposed to pesticides during the harvesting and maintenance of treated crops has long been a concern of labor, industry and regulatory agencies.[1] Re-entry studies examine the exposure of workers to pesticidal residues post-application. Re-entry exposure studies can be conducted using either passive dosimetry or biological monitoring. Passive dosimetry estimates the amount of chemical residues available for dermal and respiratory exposure using trapping devices, such as whole body dosimeters, skin patches, hand washes and air monitoring.[2] Biological monitoring estimates exposure by measuring pesticide and/or metabolite concentrations in body tissues or fluids or monitoring effects on target enzymes such as blood cholinesterase (ChE).[1] The goal of these exposure studies is to establish safe re-entry intervals to minimize exposure to workers.

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Another type of study designed to measure re-entry worker exposure to pesticides is the dislodgeable foliar residue (DFR) study. The purpose of the DFR study is to measure pesticide residues that are deposited and remain on plant surfaces after pesticide application and are available for worker exposure. Residues which can be dislodged during the performance of various agricultural tasks and subsequently deposited on human skin and clothing or inhaled are the target of DFR studies.[3] DFR data in conjunction with transfer coefficient data (surface area per time period that a worker would be in contact with foliage) are used to predict worker exposure and to establish safe worker re-entry levels.

Harvester re-entry studies are both labor intensive and costly. It has been proposed that worker exposure can be estimated using DFR studies, and several task force groups have been studying the transfer coefficient approach to re-entry exposure estimation. Several recent studies have shown good correlation between exposures predicted from DFR and harvester monitoring studies. Actual strawberry harvester exposure to abamectin ($1.36\,ng\,kg^{-1}\,day^{-1}$) compared with that predicted from abamectin DFR studies ($5.6\,ng\,kg^{-1}\,day^{-1}$) showed that DFR data are useful to conservatively estimate worker exposure.[4] Krieger suggested using DFR data as a first

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approximation for maximum exposure, and then consider clothing or other personal protective equipment which may mitigate exposure as a second tier.[5] In a study using captan in orchards, DFR was the most prominent determinant of exposure for both respiratory and dermal exposure during re-entry.[6] It must be pointed out that the results of DFR studies can vary greatly owing to:

- the physical properties of the test substance (e.g., water solubility, polarity and stability in the extraction process)
- the physical properties of the target crop (e.g., leaf surface texture, wax content and moisture content)
- application techniques such as spray additives (e.g., sticking agents, defoaming agents and tank mixes)
- meteorological conditions.

Dislodgeable soil residue (DSR) studies are used to determine exposure of re-entry workers to soil surface residues. Soil surface residues are defined as test substance residue levels in $\leq$150-μm soil particles (i.e. soil dust which can stick to a worker's skin, or can be inhaled).

2 Regulatory requirements and experimental field design

DFR studies are designed and conducted to describe the decline profile of the active ingredient on foliage and/or soil surfaces when applications are made at the proposed label rate. These surfaces are limited to those which can be touched or disturbed by workers and from which residues can be dislodged, deposited on human skin and clothing, or inhaled during the performance of field work and harvesting operations.

DFR studies are required to account for varying climatic conditions and regional techniques. Typically, DFR studies are conducted at a minimum of three sites. One source of guidance to justify geographic location of the test sites is found in OPPTS 860.1500,[7] where Table 6 of this guideline contains information listing the percentage distribution of crop production (contains data on all crops from alfalfa to watermelon) on acreage basis in each of the 13 Environmental Protection Agency (EPA) regions.

Other regulatory requirements of a DFR study include:

- sampling for 35 days' post-application or test substance residue decline through two half-life periods
- application using procedures typically found in the crop tested (e.g., ground boom, airblast, irrigation, etc.)
- well founded quality control and quality assurance program
- test sites to include extremes in meteorological conditions and major chemical usage areas; as an example, if the test substance is sold only in California, do not conduct a study in the high humidity of Florida.

Important factors need to be considered to design a DFR study:

1. Type of application equipment used: for example, using over-the-top spray boom or air blast for grapes. The sprayer should be consistent with local cultural practices for each test site.

2. Timing: applications should be made at the time of the year and plant growth stage where potential worker exposure would be at a maximum. It is critical to have a mature crop because exposure will be increased owing to the larger surface area of the foliage compared with that of an immature crop.
3. Applications should be made when the activity of field workers will result in maximum exposure (i.e., pruning, girdling, harvesting, etc.)
4. If the label allows for multiple applications of a test substance, then a minimum of two applications at the shortest spray interval are required with the decline measured from the time of the second application. The application rate should be the highest legal label rate for each test site in the lowest gallons of spray solution per acre to provide the highest potential test substance residue levels.
5. Crop maintenance issues must be defined, such as (a) no irrigation of the crop from above to wash off foliage residues, (b) application of maintenance chemicals (wash off residues and analytical interferences) and (c) mowing, weeding, grape girdling, etc., that can mechanically dislodge residues before sampling.
6. Sampling techniques must be defined and controlled. If leaf punch samples are taken, the sampling design must define the portions of the crop that will be sampled (e.g., all sides of each plant, how high to sample in trees, inner and outer leaves). Never sample a leaf by brushing with an outer leaf that may be sampled at a later date.
7. The formulation to be tested at each site must be evaluated in the light of test substances with multiple formulation usages and geographical requirements.

It is not within the scope of this article to review the EPA guidance document for DFR studies. Rather, this article will concentrate on a protocol design that will fulfil EPA requirements.

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3 Protocol design

3.1 Test system

The test systems for a DFR study are the crop leaves and, if required, the soil treated with the test substance. The variety of crop tested will be documented in the study record.

The foliar and soil treated plots and control area will be identified with markers so that the treated plots and control area do not have the same flag or marker colors.

3.2 Justification of test system

Application of the test substance to the target crop prior to harvest represents a worst-case scenario for potential exposure to workers for the crop category.

A protocol should be designed to conform as closely as possible to all EPA requirements. The test substance is a typical end-use product and application and agronomic practices accurately reflect the label and normal crop culture in the areas where the study will be conducted. Dislodging leaf material with a surfactant in aqueous

solution is the accepted convention for measuring the amount of dislodgeable pesticide residues on the leaf surface. Analysis of the <150-μm soil particle fraction of 1-cm deep surface soil is the accepted convention for measuring the amount of dislodgeable pesticide residues from soil.

Once the first spray has been applied, overhead sprinklers must not be used in the treated plot and control area under any circumstances. Irrigate only under plants for non-DSR studies.

All maintenance pesticide applications must be approved in advance and documented. Pesticides that do not interfere with analysis may be applied in order to control weeds, pathogens, and other pests if they appear in the treated plot and control area only if absolutely necessary. The treated plot and control area should be maintained with the same compound and rate and at the same time.

The crop variety and date of planting must be recorded. The variety should be one commonly grown in the area.

An accurate and complete history of pesticide use for the test site, including material applied, rate and date of application (if possible), for 3 years prior to study initiation, and also during the study, should be documented.

3.3 Materials and methods – test substance

End-use formulations should be used as the test substance. If an active ingredient is marketed in two commercial formulations, then both should be used in the study, since there may be differences in residue levels and dissipation rates, e.g., a wettable powder versus a liquid formulation. The best solution would be plots located at the same site for a side-by-side comparison. This should only be necessary at one of the sites. However, each formulation should be represented in the study unless a strong case can be made for a worst-case scenario.

The date of test substance shipment, lot number, date of receipt and method of shipment, and also the amount and container size, should be documented. The test substance should be stored in the appropriate manner and under the correct storage conditions.

Purity analysis and characterization of the test substance should be performed for each lot. A retention sample from each batch of the test substance should be archived. All unused test substance and partially empty containers should be retained until the final report is signed, unless a prior waiver has been obtained from the EPA.

3.4 Study locations

A DFR study should be conducted at a minimum of three geographical locations representative of the spectrum of climatic and crop-growing conditions expected in the intended-use areas. A crop variety commonly grown in each use area should be selected.

3.5 Plot layout

Each site should consist of two plots, as a minumum, one treated per formulation and one untreated. The untreated plot should be positioned upslope (if applicable) and upwind (at application) at least 100 ft from the treated plots to reduce the potential for contamination due to drift. All plots should be uniquely identified.

This author recommends a minimum plot size of 4 rows × 200 ft for each treated plot. Foliar sampling would occur on the middle two rows with a 10-ft buffer on each end. Soil sampling would occur on each side of the middle two rows with a 10-ft buffer on each end. The 180-ft rows should be divided into 60-ft replicates designated as A, B, and C. The untreated plot should be 2 rows × 50 ft. For tree crops, 3 rows × 14 trees should be treated. The middle row should be sampled excluding the first and last trees.

The treated plots and control area should be managed according to agricultural practice in the area. Fertilization should be made according to normal agricultural practice and documented as to when and how applied; include rate and composition. Soil preparation and other cultural practices must be noted.

Figure 1 was taken from an unpublished report, DuPont Study No. AMR 4392-97, 'Dissipation of Dislodgeable Foliar and Soil Residues of Oxamyl Following Application of Vydate L Insecticide to Tomatoes in the USA – Season 1997–1998'. This study has been submitted to the EPA and the data were used to establish and verify re-entry intervals. Data from this study will be used to provide an example of the topics discussed throughout this article.

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In this study the control plot was located upwind from the treated plot, considering the prevailing wind at the site. The distance between the control plot and the treated plot should be >1000 ft; 100 ft is the recommended minimum. The control plot was 200 × 20 ft and four rows wide with a 10-ft buffer at each end of the sampling plot.

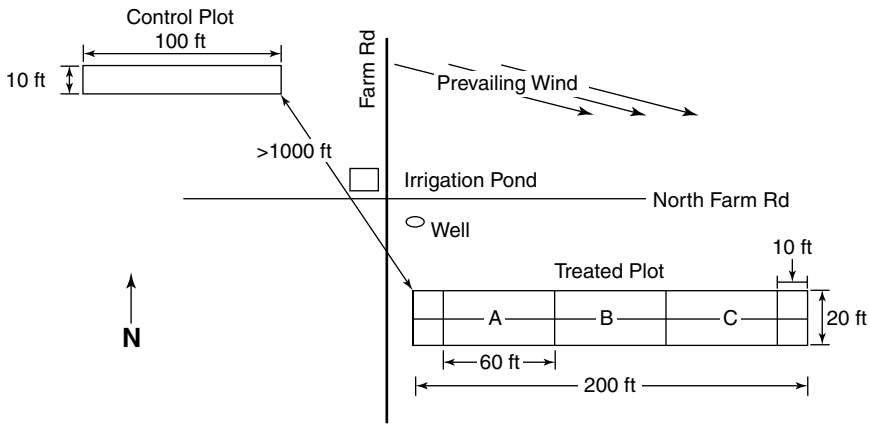


Figure 1 Typical plot diagram

3.6 Application

The type of application equipment that should be used is that normal for the region and crop. Typical equipment includes groundboom to spray on to row crops, broadcast for granular material, and airblast spray in orchards and grapes. The application should be made at the highest label rate with the minimum intervals between applications to guarantee maximum exposure. Another parameter to consider is crop maturity. It is critical to have a mature crop. Worker exposure will be increased owing to the larger surface area of the foliage.

Sprayers should be calibrated prior to each application. If, at the time of application, the wind is blowing in the direction from the treated plot to the control plot, then wait until the direction changes to prevent contamination of the control plot. Applications should occur within 1 h of mixing. Check weather forecasts to determine if wind or rain could be a problem. Airblast sprayers must be adjusted to spray through the target crop and cover the top of trees. Research sprayers often do not have the power for this job.

The actual application rate should be calculated based on output, the active ingredient concentration, and the application time or land area covered. Once the plot has been treated, the amount of product or spray volume remaining should be checked as verification of the application rate.

Target the minimum recommended spray volume per acre. The nozzle configuration should be adjusted to fit the canopy at the time of application, which is normal agricultural practice. The application sprayer should be calibrated prior to treatment. Calibration records should be maintained and submitted as raw data. Applications should be made within 1 h of mixing and applied at a time with little or no wind. The applications should be made when leaves are dry. All application parameters must be recorded. The following list gives example data collection from application:

- total spray volume mixed (gallons)
- treated area sprayed (acres)
- actual amount of formulation mixed in spray solution
- actual ground speed (not calculated)
- actual carrier rate (gallons per acre)
- actual application rate (pounds per acre)
- total spray volume remaining after application
- application date
- type of sprayer
- nozzle type
- number of nozzles
- nozzle spacing and configuration
- sprayer fan size for airblast (is it big enough for the job?)
- nozzle height
- regulator pressure
- method of tank agitation
- average crop height
- average crop width
- crop growth stage
- wind speed (on-site)

- wind direction (on-site)
- air temperature (on-site)
- percent cloud cover (on-site)
- relative humidity (on-site).

3.7 Foliar sampling

The regulations require three samples from the treated plot (one from each subplot) and a single sample from the control plot at each sampling interval. For foliage the preferred technique is to collect leaf punch samples. Leaf punch samplers are available in 5-, 2.5- and 1.25-cm^2 punch areas. Common practice requires a sample of 40–5-cm^2 leaf disks to provide a 400-cm^2 sample using both the top and bottom of the leaf disk to calculate sample surface area.

If a smaller leaf punch is used, an increased number of leaf disks must be generated for each sample. Only sample when leaf surfaces are dry from application or dew. Leaf disks are placed in glass jars for further analysis.

In the oxamyl tomato study, the DFR samples were obtained using a 5-cm^2 Birkestrand sampler (10-cm^2 disk size using the upper and lower surfaces). The sample consisted of 40 leaf disks or 400 cm^2 from each subplot. The samples were collected impartially or in a nondirected approach from the middle two rows. The plot was four rows wide and the tractor came into contact with the first and fourth rows as the application was made. The middle two rows should be undisturbed by this movement and should therefore provide a more representative sample.

For tree crops, the EPA recommends the Iwata approach.[8] In this approach, 40 punches are collected for each sample at various heights and at 45° intervals around the circumference of each sampled tree. The sampling design and technique are described below.

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3.8 Soil sampling

In most cases, if soil samples are needed, only surface samples are collected. An exception would be harvesting root crops where all residues in the top 6 in of soil would be sampled. A typical surface soil sampler is shown in Figure 2. It is the residue adsorbed on small particles (<150 μm), which could cling to moist skin, which causes the most exposure to workers. After sampling, place a flag in the center of each sampled location to mark the area against future sampling. After the surface layer has been collected, the soil is sieved to collect the fraction <150 μm and the remainder of the soil is discarded. Maintain separate sieves and collectors for treated and control plots to prevent contamination of the control samples.

3.9 Sampling intervals

The recommended sampling interval for most pesticides is 35 days after the final application or decline of test substance through two half-life degradations. For most pesticides, significant degradation takes place in the first week and the 35-day sampling

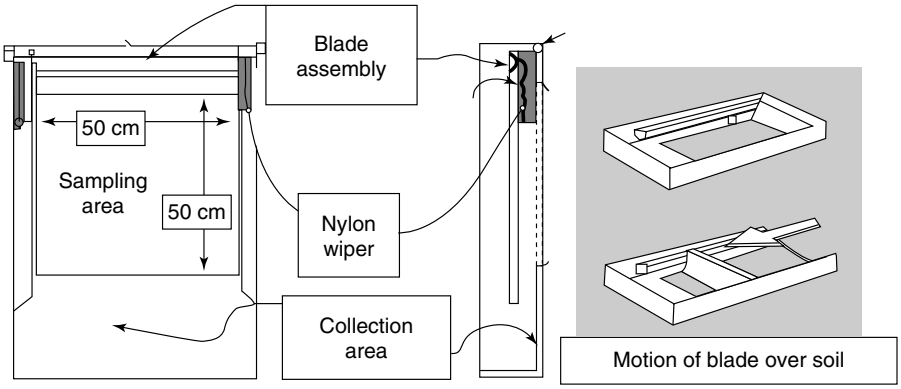


Figure 2 Surface soil sampler

period therefore encompasses several half-life periods. However, for some persistent pesticides a longer sampling interval may be needed. In the case of multiple applications, samples are collected before and on the day of each application. If the interval between applications is longer than 7 days, a sample should be collected once per week. Dissipation is monitored from the second or final application. Initially the length of time between sampling is short and may increase with time. For example, a typical scheme would be 0, 1, 2, 4, 7, 14, 21, 28 and 35 days.

3.10 Dislodging residue from leaf surface

After the foliar samples have been collected they are placed on wet ice and brought back to a facility for dislodging. The goal of the dislodging procedure is to extract the residue present on the leaf surface that is available for transfer from the leaf. Residues that have been absorbed into the plant matrix would not be available for transfer. The best practice is to dislodge the residue within 4 h of collecting the sample.

The residue is removed from the leaf surface by shaking the leaf punch sample in an aqueous surfactant solution. This allows for removal of test substance residue from the leaf surface. It does not remove residue absorbed on the plant matrix that extraction and maceration in organic solvents would release. Generally, the extraction with aqueous surfactant is performed using a mechanical shaker for a 10-min interval and is repeated to increase transfer efficiency.

Extraction conditions need to be examined during method tryout using control leaf punch samples from the study. Use of control foliage samples early in the method testing allows one to determine if analytical interferences are present. A typical dislodging solution consists of a 0.01% solution of Aerosol OT in distilled water. Other surfactants may also work and can be examined during method tryout. The use of a mechanical shaker is preferred over manual shaking. Shaking manually would become too tedious to dislodge 12 samples over a 10-min period each. A major limitation of the DFR study is monitoring residues by an aqueous dislodge procedure to describe exposure from residues by mechanical abrasion.

3.11 Field fortifications

Field recovery samples are an important part of the quality control in DFR studies. Field fortifications allow the experimental data to be corrected for losses at all phases of the study from collection through sample transport and storage. Fresh laboratory fortifications monitor losses due to the analytical phase. This section details how the field recovery process was handled in the oxamyl tomato DFR study.

Fresh oxamyl standards were prepared for each fortification event. Concentrations of 50 and 400 μg mL^{-1} analytical-grade oxamyl were prepared in a 20% acetonitrile–80% HPLC-grade water solution. The solutions were tranferred in 1-mL aliquots into uniquely identified vials so that each vial contained the correct volume of oxamyl standard to fortify one quality control sample. The vials were shipped as needed during the course of the study to each field site.

Six control sample jars of 0.01% Aerosol OT detergent in distilled water solution (200 mL each) were prepared at each site on sampling days 1, 14 and 28 by dislodging leaf disks taken from the untreated control area. Triplicate samples were fortified at each of two concentrations. Fortification levels were 50 and 400 μg of oxamyl per sample.

A 25-g sample of <150-μm control soil was placed into each of six jars for fortification. Three samples were fortified at each of two concentrations of oxamyl. Soil samples were fortified on the same days and at the same levels as the foliar fortification samples.

To fortify a sample, the label from a fortification sampling vial was removed and secured to the pre-labeled sample jar. Spike vials were individually shaken before use. The cap was discarded, the contents of the vial were poured into the sample jar and then the vial was dropped into the sample. The sample jar was capped with a Teflon-lined lid, hand shaken to mix, placed in a Kapak bag and sealed. Jars were placed immediately in storage freezers. In all cases, quality control samples were transported and stored with their corresponding field plot samples throughout sample handling and shipment to the analytical facility.

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3.12 Analysis

Before the field phase begins, it is a good practice to test out the analytical procedure. If air and water methods are available, they are good starting points to decide which extraction solvents and/or solid-phase extraction (SPE) columns will be useful to isolate the analyte from the aqueous phase. Initial extractions can be performed using filter paper as a surrogate for foliage samples. These methods will also help one decide on the instrumentation that is appropriate for the analyte; typically either gas chromatography (GC) or liquid chromatography (LC) is used.[9,10] If antibodies are available, immunoassay technology may be used for quantitation.[11]

3.12.1 Determining limit of quantitation

An important parameter to consider is the limit of quantitation (LOQ) expressed in μg cm^{-2}. The LOQ for each active ingredient will depend upon the compound

toxological properties. The LOQ of the analytical method must be low enough to detect concentrations below the no observable effect level (NOEL) in $mg\,kg^{-1}$ derived from the dermal exposure studies. The LOQ is determined using the following equation:

$$\text{LOQ} = \frac{\text{NOEL}\,(\text{mg kg}^{-1}) \times \text{body mass}\,(\text{kg}) \times 10^3\ \mu\text{g mg}^{-1}}{\text{body surface area}\,(\text{cm}^2) \times \text{safety factor}} \qquad (1)$$

In Equation (1) the body mass of a standard 70-kg person with a surface area of $2\,m^2$ ($20\,000\,cm^2$) requires a typical safety factor of either 10 or 100. The dermal NOEL for oxamyl is $50\,mg\,kg^{-1}$. In this case a safety factor of 100 is used (10 times the intraspecies variability factor and 10 times the interspecies variability factor). Substituting these parameters into Equation (1) gives an LOQ of $1.75\,\mu g\,cm^{-2}$. This is the required LOQ value based on the dermal toxicity of the test substance. However, it may be advantageous to use a lower LOQ, depending on the sensitivity of the instrumentation. In the oxamyl tomato DFR study, low fortification was performed at $50\,\mu g$ using $400\,cm^2$, corresponding to an LOQ of $0.125\,\mu g\,cm^2$.

3.12.2 Method tryout

Before the field phase begins with the first application contact, the Principal Investigator at each site requests that control matrix dislodge samples be sent for method tryout and validation. Assuming a minimum of three field sites, 15 samples per site should be adequate.

For method tryout, run a control sample and two fortifications from each site. One fortification should be done at the LOQ and the other at the highest expected residue level, perhaps 1000 × LOQ. If the recoveries are within the acceptable range of 70–120% and there are no interferences, proceed with the method validation. If interferences are present which prevent quantitation of the analyte, try additional cleanup steps with SPE or use a more selective detection method such as liquid chromatography/mass spectrometry (LC/MS).

3.12.3 Method validation

Once you have confidence that your method is adequate from the preliminary work in the method tryout, you are ready to begin the method validation. The method validation provides additional data on accuracy and precision, and confirms that there are no problems due to interference. Method validation must be completed before beginning the analysis of the treated samples from the field. The validation should test the detector's response over the expected range of concentrations from the field.

The guidelines state that a minimum of seven fortifications at three different levels be performed:

- LOQ
- intermediate (10 × LOQ)
- maximum anticipated concentration (100–1000 × LOQ)
- control sample.

To show that the method is consistent, it is best to conduct the method validation over two or three sets with different fortification levels carried out in each set. Fortify the leaf disk sample, dislodge, then add the extraction solvent and perform the extraction procedure.

3.12.4 Sample analysis

Once method validation has been completed, the treated samples may be analyzed. The method should be under control so that no additional changes will be necessary. Analysis of laboratory-fortified samples and control samples will be used to monitor the quality of the study. The purpose of laboratory-fortified samples is confirmation of the recovery efficiency of residues from the sample matrix. A minimum of two laboratory recovery samples need to run with each set. Recoveries should average 70–120%.

Another important quality measure is the analysis of the field-fortified samples. The field-fortified samples have been handled, shipped and stored in the same manner as the treated samples. Any loss of analyte in the field-fortified samples will be used to correct residue levels of the field treated samples. It is best practice to keep the storage interval as short as possible to minimize losses. The acceptable storage time will vary according to the stability of each compound in aqueous solution. A good guideline is to analyze the samples within 30 days of sampling.

3.12.5 Calculations

Dislodgeable foliar residues should be reported in units of $\mu g\,cm^{-2}$ and can be calculated using the following equation:

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$$\mu g\,cm^{-2} = \frac{\text{ng found}}{\mu L \text{ injected}} \times \text{sample volume (mL)} \times \frac{1000\,\mu L}{mL} \times \frac{1\,\mu g}{1000\,ng} \times \frac{1}{400\,cm^2} \quad (2)$$

where 'ng found' is determined from the linear relationship between concentration of analyte and instrument response, 400 cm^2 is the sample leaf surface area using the top and bottom of the leaf, and sample volume is the total volume of sample.

Degradation rates were determined for the reported data using a nonlinear regression of conventional first-order kinetic equations. The software used for this fitting procedure was Model Manager, Version 1.0 (Cherwell Scientific, 1999).

The differential first-order equation to represent the rate of degradation of the parent chemical was

$$-\frac{dM_p}{dt} = k_p M_p \quad (3)$$

This expression was numerically integrated and regressed to the experimental data using a nonlinear least-squares fitting procedure. The resulting integrated equation is

$$M_p = M_0 \exp(-k_p t) \quad (4)$$

where

M_p = concentration of parent at time t, $\mu g\,L^{-1}$ or $\mu g\,kg^{-1}$
M_0 = concentration of parent at time 0, $\mu g\,L^{-1}$ or $\mu g\,kg^{-1}$
k_p = first-order rate constant, day^{-1}
t = time after application, days.

To calculate the safe re-entry interval (REI), the margin of exposure (MOE) must be considered. Worker risk is measured as a margin of exposure and is related to how closely the occupational exposure comes to the no observed adverse effect level (NOAEL, for oxamyl 50 $mg\,kg^{-1}\,day^{-1}$). MOE is defined as

$$\text{MOE} = \frac{\text{NOAEL (mg kg}^{-1}\text{ day}^{-1})}{\text{dose (mg kg}^{-1}\text{ day}^{-1})} \tag{5}$$

For oxamyl, an MOE of >100 must be achieved in order to establish an REI which does not exceed the regulatory agency's risk concern. Dose is calculated by the following equation:

$$\text{Dose} = \frac{DFR \times Tc \times (1\text{ mg per }1000\,\mu g^{-1}) \times ED}{BW} \tag{6}$$

where

DFR = dislodgeable foliar residue, initial or obtained daily from decline curve ($\mu g\,cm^{-2}$)
Tc = transfer coefficient (10 000 $cm^2\,h^{-1}$ for tomatoes)
ED = exposure duration (8 h per day)
BW = body weight (70 kg).

3.13 Quality assurance

If the data obtained from a DFR study are to be submitted to a regulatory agency to obtain a registration, all work (excluding analytical method development and method try-out) must be conducted in accordance with applicable Good Laboratory Practice (GLP). Therefore, it is necessary to become familiar with GLP requirements before beginning the study. For a full listing of all the EPA Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) GLP requirements, see 40 CFR 160. Portions of the GLP guidelines that are critical to DFR studies are as follows:

1. Study Protocol (160.120): an approved written plan that clearly indicates the objectives and all methods for the conduct of the study.
2. Test Substance Characterization (160.105): determine the identity, strength, purity and composition which define the test substance before its use in the study.
3. Standard Operating Procedures (160.81): methods in writing that management is satisfied are adequate to ensure the quality and integrity of data generated in the course of a study.
4. Conduct of a Study (160.130): shall be conducted in accordance with the protocol. Raw data shall be recorded directly, promptly and legibly in ink.

It is required to submit protocols to the EPA for review before beginning the study to ensure that all study requirements are met.

4 Results

For the purpose of this section, the data from the oxamyl tomato DFR study will serve as surrogate data. Similar calculations could be made for any other active ingredient in a DFR study. The decline curve is shown in Figure 3. The data from the date of the last application to the interval when the residues approach the LOQ are the most significant data for the purpose of determining a re-entry interval. The long tail of additional data points just above the LOQ is not needed to establish a safe re-entry level. In a linear fit with log-transformed data, the residues at or close to the LOQ from days 5 through 28 or 35 are weighted as heavily as the data over the 1–5-day period. As a result, the initial rapid decline of oxamyl on foliage, which declines to approximately 2% of the initial value by day 5, is masked by the nature of the linear fitting routine.

A nonlinear fit weights the initial data points more heavily and gives a better description of the decline in oxamyl residues during the critical period when the residues are a concern in the evaluation of worker safety. The nonlinear curve fitting approach has been accepted by regulatory agencies for the determination of pesticide half-life determinations in soil when the decline data do not fit a linear first-order curve.

Using the nonlinear model substituting 0.915 $\mu g\,cm^{-2}$ (the DFR value from the model 1 day after application) into Equation (6) yields a dose of 1.05 $mg\,kg^{-1}\,day^{-1}$ and an MOE from Equation (5) of 47, below the required value of 100 for margin of exposure. Performing the same calculation on the day 2 data gives a dose of 0.344 $mg\,kg^{-1}\,day^{-1}$ and an MOE of 145, which is above the level of 100 required to establish a safe re-entry level. Therefore, a 2-day period is adequate to ensure worker safety. The observed values of 0.936 and 0.234 $\mu g\,cm^{-2}$ for days 1 and 2 match closely the values predicted by the model.

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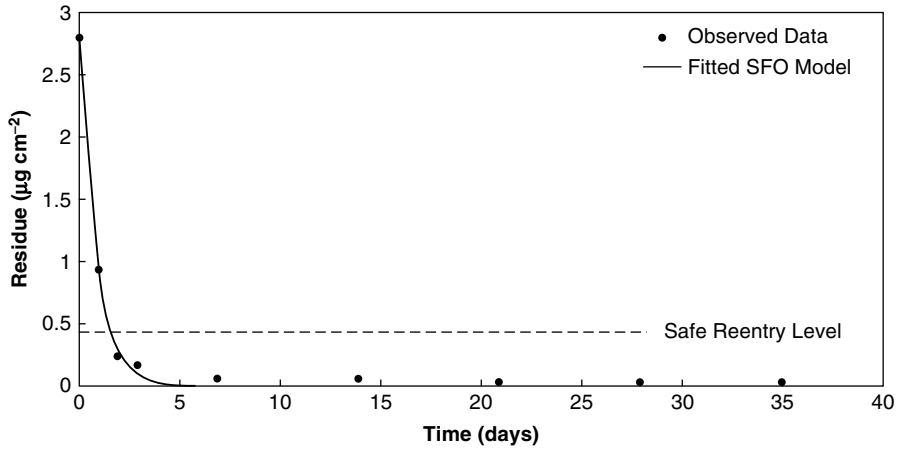


Figure 3 DFR decline curve for oxamyl applied to tomatoes in California

5 Recommendations

DFR studies are designed and conducted to describe the decline profile of the active ingredient on foliage and/or soil surfaces when applications are made at the proposed label rate. Surfaces are limited to those which can be touched or disturbed by workers, and from which residues can be dislodged, deposited on human skin and clothing, or inhaled during the performance of field work and harvesting operations.

Other regulatory requirements of a DFR study and important factors that need to be considered to design a DFR study are listed in Section 2.

In addition to the decline data, the following list of tables should be included in the report:

- general plot description
- sprayer specifications
- environmental conditions and application data summary
- chain of custody dislodging solution samples
- weather data and comparative historical data
- method validation data
- recoveries of field-fortified samples
- recoveries of concurrent laboratory fortifications
- chromatographic conditions (example chromatograms should be included with the figures)
- dislodgeable foliar residue levels.

References

1. N.J. Simcox, J. Camp, D. Kalman, A. Stebbins, G. Bellamy, I.C. Lee, and R. Fenske, *Am. Ind. Hyg. Assoc. J.,* **60**, 752 (1999).
2. US EPA, Occupational and Residential Exposure Test Guidelines, OPPTS 875.1000, Background for Application Exposure Monitoring Test Guidelines, Environmental Protection Agency, Washington, DC, p.14 (1996).
3. US EPA, Occupational and Residential Exposure Test Guidelines, OPPTS 875.2000, Background for Postapplication Exposure Monitoring Test Guidelines, Environmental Protection Agency, Washington, DC, p. 4 (1996).
4. C.L. Lanning, T.A. Wehner, J.A. Norton, D.M. Dunbar, and L.S. Grosso, *J. Agric. Food Chem.,* **46**, 2340 (1998).
5. R.I. Krieger, *Toxicol. Lett.,* **82/83**, 65 (1995).
6. J. de Cock, R. Hederick, H. Kromhout, J.S.M. Boleij, F. Hoek, H. Wegh, and E.T. Ny, *Am. Ind. Hyg. Association J.,* **59**, 166 (1998).
7. US EPA, Residue Chemistry Test Guidelines, OPPTS 860.1500, Crop Field Trials, Environmental Protection Agency, Washington, DC, p. 70 (1996).
8. Y. Iwata, J.B. Knaak, R.C. Spear, and R.J. Foster, *Bull. Environ. Contam. Toxicol.,* **18**, 649 (1977).
9. G.F. Antonious, M.E. Byers, and J.C. Snyder, *Pestic. Sci.,* **54**, 61 (1998).
10. J. de Cock, R. Hederick, H. Kromhout, J.S.M. Boleij, F. Hoek, H. Wegh, and E.T. Ny, *Am. Ind. Hyg. Assoc. J.,* **59**, 158 (1998).
11. J.A. Itak, M.Y. Selisker, C.D. Root, and D.P. Herzog, *Bull. Environ. Contam. Toxicol.,* **57**, 270 (1996).

Best practices to conduct spray drift studies

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1 Introduction

The intent of this article is to provide an overview of methodology and materials for sampling airborne pesticide spray and spray collection on surfaces by sedimentation deposition and impaction after release from field sprayers. The term 'pesticide' will be used as a general term to represent chemicals such as herbicides, insecticides, fungicides and related products.

Other agricultural and biological chemicals can also usually be sampled using the methods described in this article.

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Spray drift is defined for this topic by the National Coalition On Drift Minimization (NCODM) as 'The physical movement of pesticide through the air at the time of pesticide application or soon thereafter from the target site to any non- or off-target site'. Secondary drift, defined by NCDOM as 'vapor drift or subsequent dust and particle movement after the application', is only partially addressed, although most key principles discussed will still also apply to such secondary movements.

The US Environmental Protection Agency (EPA) instituted formal spray drift study guidelines (201 and 202 Sub Div. R) in 1985 in order to assess off-target spray deposition that could affect aquatic and terrestrial ecosystems. The FIFRA 88 data call-ins indicated EPA's interest in drift data for each formulated product for each use pattern. The cost of generating the required studies was estimated to be well over $1 billion in 1990. The high cost to the industry, the limited number of facilities capable of the work and the expectation that drift would be independent of active ingredient prompted registrants to form a joint venture to supply data. The EPA supported the effort in order to obtain a larger and more scientifically thorough, consistent and useable drift database than it would have obtained through the independent efforts of registrants. The Spray Drift Task Force (SDTF) was formed expressly to meet the EPA requirements for data on spray atomization and drift following four major application patterns (aerial, ground, orchard and chemigation). The SDTF has conducted and submitted to EPA

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numerous studies during the past 10 years and has become an important focus of spray drift knowledge and study expertise. Current EPA testing guidelines, now numbered 840.1000–1200, are being updated in light of EPA's acceptance of SDTF protocols and data.

The goal of spray drift sampling is to obtain representative samples of the application being investigated while minimizing any bias introduced by the methods employed to collect those samples. Collected samples are used to investigate whether pesticide products are present in the environment and, if present, at what rates relative to the amount of product(s) applied to the spray area (the field, forest or other spray area).

This review is structured to address tracer (or test) materials (dyes, metals, salts, active ingredients and other compounds), sampling locations, sampler types (active and passive; artificial or natural), meteorology and performance criteria (stability, extractability, recovery, levels of detection). Each section includes a literature review of many different approaches that have been used with varying degrees of utility for field studies. Following the literature reviews, recommendations are given for methods with which the authors have had particular success. However, the reader is encouraged to consider carefully his or her own needs before determining the most appropriate method for a given study type. Studies that need to be conducted according to Good Laboratory Practice (GLP) standards will require specific protocol, standard operating procedure and quality assurance issues relating to study design, techniques and data interpretation.

2 Study designs

2.1 Study objectives

Spray drift studies may be conducted to quantify off-target deposition or drift or to determine spray coverage and distributions within a particular application area. A quality study involves four key elements:

- selecting sampling, site and weather station locations relative to the application area
- selecting analyte and compatible collection devices appropriate for the objectives of the study and meeting the stability requirements
- establishing field procedures and quality control procedures for application rate, sample collection and handling
- assessing collection efficiency, stability, extractability and precision and bias (e.g., detection level).

Data collected in drift studies may later be interpreted in risk assessments in conjunction with toxicity data for specific sensitive areas. For example, a risk assessment for determination of appropriate mitigation (if necessary) may include field study data on exposure risk from drift, along with information on other routes of exposure (e.g., dislodgable residues, runoff, etc.) and toxicity data from laboratory and/or field study models. The results of such an assessment may be used to estimate whether a given exposure represents a hazard to any specific entity or ecosystem.

Exposure of organisms to pesticides occurs through contact or inhalation. Inhalation exposure can be assessed using some of the active samplers discussed in the previous section, for example air samplers mimicking respiratory systems. Contact exposure can be assessed using samplers that represent collection by horizontal or vertical surfaces, or combinations of these orientations. This article addresses only the first part of this process, i.e., consideration of techniques for sampling sprays in the environment.

2.2 *Tracer materials*

Tracer materials are defined as any product included in the test substance that can be recovered analytically for determining the drift from the application. This may be the active ingredient in an actual tank mix, or it may be a material added to the tank mix for subsequent detection. The selection of an appropriate tracer for assessing deposition rates in the field is critical to the success of a field study. Tracer materials such as low-level active ingredient products, colored dyes, fluorescent dyes, metallic salts, rare earth elements and radioactive isotopes have been used with varying degrees of success in the field. An appropriate tracer should have the following characteristics:

- stability under the pH, sunlight, humidity, temperature and storage conditions of the study
- detectability at appropriate levels for the study objectives
- low toxicity and low background levels in the environment
- compliance with label and legal requirements
- solubility in the tank mix; if not soluble, uniformity of the tracer must be maintained
- ease of analysis
- low possibility of volatilization.

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When a tracer is considered, it is important to evaluate its performance with respect to these criteria, especially stability during exposure and storage/analysis. Normal practice involves conducting weathering tests where field collectors are treated with known amounts of the tracer and an assessment is made of weathering, extraction and storage stability under conditions pertaining to the intended use. The characteristics of the tracer allow it to be applied uniformly over the application area. Typically, application monitors are used to verify both the application rate and the uniformity of the application.

2.2.1 Active ingredient tracers

Active ingredient tracers or test substances can be quantified using gas chromatography (GC) and high-performance liquid chromatography (HPLC) analyses.

Where there is particular interest in a specific product, it is useful to consider using that active ingredient as the tracer. Active ingredient tracers are also useful for generic drift studies,[1] such as those of the SDTF. The SDTF found that the active ingredient tracers diazinon and malathion could be used effectively to measure drift to very low quantities without degradation under field conditions. The active ingredients were used at low rates of only 10% of the normal commercial use rates, providing the same

trends as when tests were conducted at full commercial use rates. The advantage of a low use rate is that the same test area may be sprayed several times for different experiments without exceeding the label use rate for these products.

2.2.2 Fluorescent dyes

Colored and fluorescent dyes have the advantage of being relatively cheap and easy to use. Standard procedures are available for detection of the dyes using colorimeters and fluorimeters. Some of these instruments can be used in the field to analyze samples as they are collected following exposure to the dyes. Fluorescein has been widely used for studying spray deposition within and outside canopies.[2]

A problem that has been encountered with many dyes is that they tend to degrade in sunlight.[3–5] The SDTF studied fluorescent dyes in various laboratories and found that Eosine OJ and Tinopal CBS-X were relatively stable. However, when the same dyes were used in the field, it was discovered that they were not stable under warm and humid conditions owing to aqueous photolysis. The SDTF therefore decided to use dilute active ingredient and metal tracers that were more stable.

Some fluorescent dyes are more stable than others.[6] For example, our experience suggests that Pyranine 10G is sufficiently stable if samples can be collected within less than 30 min. For wind tunnel measurements of spray drift, success has been obtained with Green S.[7]

2.2.3 Metals and other tracers

Metals such as copper or manganese have been successfully used for field drift studies, allowing good detection levels and stability under most conditions. In Germany, extensive drift studies were conducted using copper as a tracer.[8] In the USA, researchers at the United States Department of Agriculture, Wooster, OH, have also used metals as tracers in field studies.

2.3 Selection of sampling locations and site preparation

The layout of a field study site needs to be established based on the study objectives. Typically, several lines of sample will be laid out in the downwind direction from the application area, perpendicular to the sprayer travel direction assuming a crosswind normal to the application direction. Three or more parallel lines will provide useful information on spray deposition in the sampling area. If wind directions may be variable, these lines can be set up in various directions radiating outwards from the application area.

The American Society of Agricultural Engineers (ASAE) has developed standard test method criteria for consideration in setting up field studies, ASAE Standard S561.[1] Researchers are strongly advised to refer to this standard when designing field drift studies. The standard also describes the setup of the field sprayers, the monitoring procedures for meteorological conditions, sampling media and other factors affecting drift studies, and the documentation that should be taken in field studies (test site layout, crop details, adjacent structures, etc.).

The spray line (the distance along which an application will be made) must be longer than the sample lines, to ensure that appropriate sampling rates are included. Typically, the required length will be 2 × sin 30 × maximum sample distance, where 30 is the degrees of wind azimuth mean variation from sample line azimuth. The distance for sampling will depend on the application type and release height, droplet size, wind speed, detection levels, study objectives (with respect to distance) and limitation of the available field locations. Since they will affect the airflow, obstructions (e.g., buildings, tall vegetation, etc.) should usually be avoided. They generally require 10–20 times their height downwind to return wind patterns and turbulence to near ambient conditions. Collection stations are usually spaced with geometrically increasing width from each other as distance increases from the application area in order to sample the deposition decay curve.

The generic protocols developed for SDTF field studies with EPA input included the following recommendations for location of field collection stations:

- The application length was 2000 ft; this width was sufficient to allow four parallel spray swaths (i.e., 180 ft for four swaths each 45 ft in the aerial studies).
- Collection stations were located at various distances from the point of application up to 2600 ft in the aerial studies, 1300 ft in the ground application studies and 1000–1500 ft in the orchard studies.
- All distances were measured from the downwind edge of the effective swath located on the downwind side of the application zone.
- Three parallel collection lines were located 50 ft apart and perpendicular to the application line. This pattern allowed three replicate measurements to be made at each sampling distance.
- Samples were also collected within the application area (to verify on-target application rates) and upwind of the application area (to verify that drift did not occur in the upwind direction).

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2.4 *Sampling devices*

Sprays and dusts can be sampled using various different devices and collection media. Collectors may be active or passive, depending on the type of information that is needed from the study. For example, if information is needed on airborne spray concentrations, active collectors may be most appropriate since these can sweep the air of material and the data collected can be expressed as mass flux, total concentration or in other units. On the other hand, passive collectors such as flat cards may be more useful if information is needed on spray deposition on an aquatic or terrestrial surface. Collectors may also be designed to involve natural surfaces such as vegetation of interest in a particular study (which may be analyzed for levels of damage from a given active ingredient pesticide), or simulated foliage made from materials from which it may be easier to extract the tracer materials. All sampling devices must be handled with care to prevent contamination of low residue samples by high residue samples. Sampling devices should be handled with disposable gloves that are replaced at each sampling distance. Specific individuals should be assigned to sample specific areas so that transfer of residues by study personnel may be avoided.

2.4.1 Active collectors

(1) Air samplers. The most common type of active sampler is the air sampler.[9,10] Air samplers may be high or low volume, and may or may not be isokinetic (to balance the input rate with that of the air movement conditions at the time of sampling). Active samplers require power (usually from batteries or generators) to cause suction or rotation. They may be oriented to provide collection representative of human respiratory systems (e.g., downward for the nose or forward for the mouth). They may be oriented to achieve maximum intended collection for worst-case assessments of airborne spray material. These types of collector require sampling media, such as glass-fiber filters, foam pads, sorbent resins, charcoal or other material that can be removed and washed for extraction of tracer materials in the laboratory. With continual drawing through of air, some material may be lost from this kind of sampler, especially if the material is volatile. This should be determined for the product(s) being sampled in the field, to ensure that any potential losses are accounted for in the data analysis. More information on assessments of performance of sampling with respect to precision and bias is provided later in this article.

High-volume air samplers are usually preferred where low concentrations of pesticides are being measured ($\mu g\ L^{-1}$, ppb range) over extended time periods of hours or days. They usually require power sources such as direct electric connection or a generator. Low-volume air samplers have the advantage of being more portable, are battery-operated and are usually used for measuring medium to low concentrations ($mg\ L^{-1}$ to $\mu g\ L^{-1}$) over shorter time periods up to a few hours. Low-volume personal samplers are typically used for air monitoring or measuring worker exposures to pesticides at air flow rates up to 5 $L\ min^{-1}$ for medium/low concentration levels ($mg\ L^{-1}$ to $\mu g\ L^{-1}$) over a time period up to a few hours. Standard Industrial Hygiene Association approved samplers are recommended because they have established acceptance and utility.

(2) Rotary impingement samplers. High- and low-volume air samplers usually only provide information on spray concentrations in the air. Other active samplers may also provide information on droplet size spectra and droplet densities. For example, Teflon-coated slides are used in rotary impingement devices for assessments of droplet size near the ground in mosquito adulticide operations.[11] Magnesium oxide-coated slides have been used for similar assessments in other vector control and spray research.[12] These slides are prepared by burning strips of magnesium ribbon below glass slides (typically very narrow for collection of small droplets). When droplets impact on the slides within rotary samplers, they leave a crater that is 1.16 times the diameter of the original droplet. A particular advantage of this approach is that the spread factor is constant for a wide range of droplet sizes and spray materials.[13] This is not the case with many other collection materials such as water- and oil-sensitive paper [see Section 2.4.2.(1)]. Correction factors for collection efficiency based on droplet size were proposed by researchers in Europe, who used this technique for extensive field research in applications of aerosol sprays.[14]

Rotary sampling devices called 'Rotorods' have also been widely used for sampling airborne sprays.[15,16] These devices are similar to the rotary samplers described above, but do not use Teflon or magnesium oxide slides for spray collection. Rather, droplets

are swept from the air passing the collector by two vertical probes. These are then analyzed in the laboratory for volume of spray material. The collection efficiency of these samplers was addressed in research sampling airborne spores.[17]

The present authors have had experience using rotary samplers for field studies involving relatively small droplets for vector control applications and for the measurement of droplet size at far-field distances. When using magnesium oxide slides, the spread factor for droplets varies from 0.75 for crater diameters up to 15 μm, to 0.8 for 15–20 μm and 0.86 for crater diameters above 20 μm.

(3) Cascade impactors. Cascade impactors provide information on particle or droplet size spectra within airborne sprays. The air is drawn through a series of chambers that allow sequential separation of different particle sizes based on their different velocities and masses. This type of collector is not as widely used as the sampling devices discussed previously because they are relatively difficult to operate and are expensive. Further information on this and other types of sampler for spray research can be found in the literature.[18,19]

2.4.2 Passive samplers

The previous section described active samplers where the air is swept of particles using mechanical mechanisms. This section describes passive samplers that do not move, but collect material that deposits by impaction or sedimentation deposition. These types of collector are the most common type for field studies aimed at assessing exposure of aquatic and terrestrial organisms to pesticides.

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(1) Sediment deposition. Horizontal surfaces collect particles primarily by sedimentation deposition, sometimes referred to as 'fallout'. These can provide valuable information relating to exposure for horizontal surfaces in the field such as water and ground. Most studies measuring spray performance within the application area, and/or outside this area by drift, include horizontal collectors. The most common types of such collector include flat papers, cards and plates. Water- and oil-sensitive papers have been widely used for looking at the uniformity of spray coverage, coefficient of variation, droplet densities and approximate droplet size within a spray block.

Water-sensitive papers are readily available in most countries and provide a convenient system for visually assessing spray drift performance. These papers are coated with bromoethyl blue, which turns from yellow to blue when contacted with water.[19,20] However, since any water can cause this change in color, care needs to be taken to prevent accidental exposure to sources of water other than the pesticide. Such cards do not work well under humid conditions, and are not appropriate for sampling droplets with diameter below 15 μm. Quantitative estimates of droplet size distributions must take account of the exponential increase in droplet volume as the droplet diameter increases. As droplets strike the paper, the liquid spreads over the surface and leaves a stain with a size that is dependent on the volume of the droplet. The apparent droplet size will be greater for large droplets than for small droplets, and the size determination must be corrected to avoid bias.

Oil-sensitive papers can be used for sampling oil-based sprays. These white papers turn black when contacted by oils.

Various tools are available for analyzing these papers using microscopes and/or image analysis systems. Water- and oil-sensitive papers can provide relative information on deposition for spray applications. Since they are affected by factors related to collection efficiency, spreading of droplets, errors from overlapping or sideways-impacting droplets, they should not be used for assessing absolute data. They are not generally able to resolve sufficiently small quantities of material to be of use in drift studies beyond crude very near-field assessments.

Other paper collectors that have been used to assess droplet size and distribution patterns include cards such as Kromekote.[12,21] This was one of several types of collector that provided information on spray deposition in the field. α-Cellulose samplers are fibrous in nature, and include a vertical component to their aspect. This type of collector, along with Mylar cards and other types of card samplers, are often used to provide information on spray coverage as amount of material per unit surface area.

Among horizontal collection media, the present authors have had excellent experience using absorbent material such as α-cellulose and filter papers, which have reasonable collection and retention performance for active ingredient and other tracers. The SDTF effectively used 0.1-m^2 α-cellulose cards to provide deposition data for modeling. These cards can be placed on suitable mounting surfaces to avoid contamination when placed on the ground or other surfaces. The cards can then be placed on the ground at appropriate sampling locations, and collected after exposure in the field. Cards may be more efficiently analyzed if cut in sections for extraction. Care should be taken if placing the cards in the wake of objects or vegetation, since this may reduce the exposure compared with open locations. The collection efficiency may also differ if the cards are located at heights above the ground since the streamline followed by the air carrying any drifting particles may differ with changes in location and height relative to the droplet locations, velocities and trajectories.

2.4.3 Impaction

Vertical surfaces collect spray primarily by impaction. Plants and other entities with a vertical component will collect some material by impaction, and typically also by deposition, as discussed in the previous section. While horizontal collectors will tend to collect all or most material that falls out on to them, vertical collectors will have collection efficiencies that are more closely related to their physical characteristics, especially collector diameter. The SDTF used α-cellulose cards oriented perpendicular to the ground and strings made of cotton or Teflon in its field studies[1] to assess spray volumes at locations above the ground. Many other researchers have used strings for assessing airborne spray volumes in drift studies.[22,23]

Vertical surface collectors can readily provide information on relative drift (e.g., the amount of drift from one field trial compared to another). However, it is difficult to obtain absolute data unless the precise collection characteristics are known for the droplet size spectrum at the point of spray collection, wind speed and air turbulence intensity.[24] The SDTF conducted studies in wind tunnels to compare the collection efficiency of different types of drift collector used in its field studies. These studies showed that collection efficiency on strings was several orders of magnitude higher for ~0.8-mm diameter cotton string than for 2-mm diameter polyethylene line and vertical α-cellulose strips or squares. The higher collection efficiency for the cotton

string was attributed to its narrower diameter and fibrous nature (which produced a very high surface area to volume ratio).

2.4.4 Bioindicators

Many researchers have used natural or artificial vegetation (e.g., artificial Christmas tree foliage or pipe cleaners simulating narrow leaves) for sampling spray drift and deposition relating to specific targets. Natural vegetation can provide information on exposure effects using bio-indicators such as potted or free-growing plants. With these collectors, the amount of damage can be compared with control plants that were not exposed in the field study. The interpretion of the results may be complicated by the difference between the sensitivity of field plants and potted plants that are grown under protected, sheltered conditions in greenhouses. The interpretation should also consider the possibility of recovery from any damage over time, and effects on the population (at least multiple rows) should be evaluated rather than effects on individual plants. Bio-indicators may also be affected by secondary spray movements such as re-volatilization of active ingredient, or wind-borne contaminated particles or dust. Primary drift is the off-target movement of materials at the time of application or soon thereafter, whereas secondary movements occur later, following initial droplet or particle deposition. Bio-indicators and air samplers have sometimes been used for studying secondary and/or primary drift.[25,26]

Several researchers have used natural collectors for drift and deposition studies in the field. Research into propanil movements from applications to rice crops has included bio-indicators along with other types of collector.[26] Potted cotton plants were assessed for leaf damage, and residues were determined from chromatographic analyses of plant leaves. Other researchers have also used vegetation surfaces to assess off-target spray movements;[27–29] however, a weakness in using plants as collectors is the difficulty in quantitatively extracting the pesticide residue from the plant surface.[30] The pesticides may become absorbed into the plant, or be bound to the surface or physically trapped, so that extraction is not quantitative. Both biotic and abiotic degradation/metabolism processes could occur so that the definition of residue and the development of analytical method become difficult. Biological indicators are only of use for evaluations of effects of specific chemicals on specific nontarget organisms, and have collection efficiency variability; hence they are not very useful for generic studies. Furthermore, the selection of biological indicators is somewhat subjective for a given study type. For these reasons, the SDTF studies and GLP studies in general have not used these types of sampler for assessing off-target spray movements.

The present authors have used bio-indicators for field drift studies where there is a need for information on damage to a particular sensitive species from a particular herbicide. In this case, bio-indicators were used with other types of collector for measuring primary and secondary movements of herbicide following aerial and ground application. Greenhouse-raised potted plants were selected for uniformity and placed in the field at the various drift sampling locations, as well as upwind of the application area (field control sample). To assess the effects of the nominal application, plants were also placed within the swath, and unexposed, control samples were retained in the greenhouse. The plants were transferred to a greenhouse following

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exposure in the field to drift and secondary movement phases. Separate plants were used for each exposure period up to 2 days following application. Damage levels were determined based on visual symptoms of foliar damage for several days following exposure and collection. The data were more qualitative than quantitative because information was not readily available to relate each level of injury to specific amounts of active ingredient. However, this type of information can also be developed if needed by fortifying laboratory plants with known amounts of active ingredient at various levels from the minimum amount, causing visible damage up to 100% of the application rate.

We have also had success using string as a collector for airborne spray. Strings are especially useful for assessing vertical spray profiles (concentration of active ingredient at different heights above the ground) in orchard airblast studies, where the spray is applied in an upwards direction (as opposed to downwards from most aerial, ground and tower or wrap-around sprayer orchard applications). For example, in the SDTF field studies, strings were located between different tree rows to quantify the vertical spray profile in the wake of increasing rows of trees. The strings were cut into sections for establishing the vertical profile against height above the ground. A string of 0.8-mm diameter (for high collection efficiency as described previously in this section) and minimal color (to avoid interference with GC analysis of active ingredient tracers) was selected. String has an advantage over smooth collectors such as polythene line in that, being fibrous in nature, string tends to absorb and retain droplets more effectively, whereas they may run off smoother types of collector, particularly near the target area where there will be high loading of drift droplets.

2.5 Field data requirements

Meteorological conditions should be monitored at frequent time intervals in the field at appropriate locations (e.g., in the downwind sampling area and if possible at a second location, e.g., in the application area or immediately upwind of that area). Wind direction is critical when interpreting the data collected on field sampling media, since even a small change in the wind direction relative to the sample line can cause a large change in the effective distance of the furthest sampling locations. A cosine correction can be applied to correct these distances based on actual wind direction. If the deviation was more than $\sim 30^{\circ}$, it may not be appropriate to use the data even if they are adjusted, unless there is confidence that a sufficiently long application area was used to ensure appropriate collection at a given distance. For meteorological monitoring, the following are recommended:

- dry bulb ambient air temperature within $\pm 0.4\,^{\circ}$F at a height of 6 ft above the ground
- wet bulb air temperature within $\pm 0.4\,^{\circ}$F at a height of 6 ft above the ground and/or relative humidity within $\pm 2\%$ at a height of 6 ft above the ground
- horizontal wind speed ± 0.5 mph at a height of 6 ft above the ground (or greater for orchard studies, as appropriate based on the canopy height)
- horizontal wind direction $\pm 2^{\circ}$ at a height of 6 ft above the ground (or greater for orchard studies, as appropriate based on the canopy height)

- sky conditions (cloud cover, etc.) and any unique meteorological conditions should be documented
- barometric pressure and solar radiation may also be measured if instrumentation is available for such measurements.

3 Performance criteria

Researchers should be aware of, and account for, factors that can affect the performance of field studies with respect to precision, bias and possible error influences. The major factors affecting collectors, tracers and analytical approaches have been discussed elsewhere in this article. In summary, these are collection efficiency, stability and detection levels, respectively. Collection efficiency (or impaction parameter) for field samplers is related to particle/collector diameter and wind speed relationships, as summarized by the following equation developed by May and Clifford:[31]

$$P = f[(pV_0d^2)/(18Vl)] \quad (1)$$

where P = impaction parameter, p = droplet density (kg m^{-3}), V_0 = droplet velocity (m s^{-1}), d = droplet diameter (m), V = air velocity (m s^{-1}), l = target collector dimension (m) and f = function (mathematical symbol).

Collection efficiency is a measure of the amount of material collected by the sampler relative to the amount of material to which the sampler was exposed. Collection efficiencies for many types of samples can be obtained from literature references. If not available in the literature, collection efficiencies can be obtained by comparing the amount collected by the sampler with the amount collected by samplers with known collection efficiency (e.g., nominal 100% for isokinetic samplers). Alternatively, the collection efficiency can be determined by measuring the amount of material collected in a low-speed wind tunnel or spray chamber relative to the release of a known amount of material. Some samplers have collection efficiencies below 100% (e.g., wide collectors sampling small droplets), while others may exceed 100% if they sweep the air of more material than passes a given location based on sampling area alone (e.g., high-volume air samplers).

Stability of tracers is an important issue that can affect measured deposition rates. Metals and active ingredient tracers are usually more stable than dyes. Stability can be assessed in the field by spiking the sampling media with known amounts of tracer in a tank mix, and then leaving the samples exposed to weathering in the field (sunlight, wind, humidity, etc.). The samples are then analyzed for recovery of material relative to the fortified rate. Fortification samples should be prepared at several rates within the range that will be covered by the study. A stability-related issue pertaining to air samplers is the ability of the sampling media (e.g., filters) to retain tracer material while the sampler continues to draw though air. This should also be assessed in laboratory studies where the air sampler is operated for different periods of time and samples analyzed for amount of tracer against operating time.

Detection levels are affected by the ability to extract the tracer from the sampling medium using available solvents in the analytical laboratory, and by the level of

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quantitation of the instrument used to analyze the samples. These should be known for the technique that is used for a given study and can be provided by most analytical laboratories.

The success of any study is dependent on the selection of appropriate techniques, test sites, study design and replication. Considerations for many of these criteria in air sampling studies were discussed by Sava.[10] For example, it is recommended that for indoor sampling work, high-volume samplers should be vented out of the dwelling to prevent air recirculation errors. Rooms with smoke or gas appliances are not recommended since suspended particles or gases may be trapped in the sampling media, interfering with the analyses. When working outdoors, air intakes should be positioned to avoid drawing in air from generator or other exhausts, smoke or other nontarget air contaminants. Samplers should be protected from rain and direct spray using shelter hoods and using horizontal mounting arrangements. Timers may facilitate automatic sampling in remote locations. After collection, samples should immediately be placed in labeled, sealed containers in freezing, dark conditions to minimize breakdown, and all handling should be done with clean protective gloves, tweezers, etc., to minimize contamination exposure.

4 Covariate study designs

Off-target spray movements are a function of application scenario and meteorological effects. As explained earlier in this article, other variables are also involved, such as canopy and collector characteristics. Since meteorological variables continually change, the ideal way of comparing treatments is to apply all treatments simultaneously. This is almost impossible, since each treatment would require different tracers and sprayer tanks and booms making the applications within a very short time frame. The covariate approach provides a possible solution to this by always applying two treatments almost simultaneously. One treatment always involves the same application scenario (i.e., the test substance and application parameters remain constant), while the second treatment includes a change of those variables being studied. These treatments are referred to as the 'standard' and 'variable' treatments, respectively. Replication is a normal practice to repeat each treatment pair; however, with possible changes in meteorological variables, the repeat treatment pairs cannot be assumed to be 'true replicates' in the normal use of the term. A covariate analysis is calculated using the standard treatment as the covariate for facilitating comparisons among treatments without the confounding effects of meteorology. The data can also be processed to show the effects of meteorological differences between the standard treatment database, for example using a multiple regression analysis of all meteorological variables (temperature, relative humidity, wind, etc.) with off-target deposition from the standard treatments.

It should be noted that the analysis of covariance assumes a linear relationship between the standard value and the variable value, for a given treatment, and assumes independence between an effect of a given treatment and the value of the standard. The relationship between these may be more complex in many drift studies. Therefore, the standard treatment should be near the median of the variables being investigated. The only alternative involves a great deal of patience and time to obtain very similar

meteorological conditions, generally requiring multiple days, even in areas of predictable weather patterns.

The SDTF used covariate study designs in its field research.[1] For aerial applications, a specially modified aircraft was used to make the applications in the field. This aircraft had two sets of booms, tanks, pumps, nozzles, etc. One boom was always fitted with the same type of nozzle (D6-46), which allowed the application of a standard treatment that was always the same (using diazinon as the active ingredient tracer). The other boom was fitted with different nozzles for different applications of the variable treatment (usually using malathion as the tracer). For orchard and ground spraying studies, an application with a standard sprayer was made within a few minutes before or after an application with the variable sprayer. By analyzing each sampler for both active ingredient tracers, the data could be interpreted as follows. Differences in drift between the standard treatments could be attributed to meteorological effects, since the application parameters were always the same, while wind speed, wind direction, temperature, relative humidity and air stability differed at different times of the day and on different days. Differences between the standard and variable treatments were due to both application and meteorological conditions, and when analyzed using treatment pairs, application parameter effects could be assessed.

For setting up field studies using standard reference sprayers that are intended to produce significant drift rather than simulate a typical application, it is recommended that the configurations described in ASAE Standard S561[1] be used where possible and appropriate. This standard describes in detail the setup and execution of such studies. Reference sprays are used primarily to study the effects of environmental conditions or effects of orchard canopy cover on spray patterns. For example, the reference spray for aerial applications may include applications at 30 psi pressure through at least 30 size D6-45 nozzles mounted at 45° down and back with a boom length of 70% and a flight speed of 120 mph. Ground application reference sprays are based on using 8004 nozzles at 40 psi with a boom height of 18 in above the ground, a 20-in nozzle spacing and a sprayer speed of 5 mph. Orchard application reference sprays include rearward-facing D6-25 nozzles mounted on a vertical boom at 1, 2, 3, 4 and 5 m above the ground with an operating pressure of 60 psi and a sprayer forward speed of 2.5 mph. These application conditions typically generate a significant amount of drift that is collected at various sampling heights to determine vertical drift distributions.

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5 Summary

The variety of collection devices, tracer materials and study layouts that can be considered when designing studies investigating spray drift and deposition is huge. Different approaches will be appropriate for different objectives, budgets and geographical locations. This article has provided a review of the most common approaches that have been used with varying degrees of success for such studies in the field. Several industry standards and test guidelines are available when designing field application studies. It is important to be aware of the limitations of the methodologies that are selected for a given study, to ensure that the data collected are interpreted correctly.

Other studies may be conducted in conjunction with the field studies to facilitate the interpretation of the data for application in decision making. These might include atomization studies to measure the droplet size spectra that pertain to the field application scenarios, and using the same nozzles, angles, pressures, air velocities and tank mixes that were used in the field, to measure emission droplet size spectra in a wind tunnel.[32] These data are useful for assessing the drift in relationship to emission droplet size spectra and for modeling drift based on droplet size and other parameters.[33] Toxicological data for the active ingredient products of interest will be useful in interpreting the possible effect of a given measured amount of drift on specific aquatic and/or terrestrial organisms, for example for assessing protective measures using models.

References

1. ASAE, 'Procedure for Measuring Drift Deposits from Ground, Orchard and Aerial Sprayers,' Standard No. S561, American Society of Agricultural Engineers, St. Joseph, MI (1997).
2. H. Koch and P. Weisser, *Aspects Appl. Biol.,* **57**, 357 (2000).
3. D.B. Smith, D.D. Plummer, F.D. Harris, and E.E. Picket, *Trans. ASAE,* **26**, 1308 (1983).
4. W.E. Yates and N.B. Akesson, *Trans. ASAE,* **6**, 104 (1963).
5. J.S. Yuill and J.P. Secrest, *J. Econ. Entomol.,* **59**, 719 (1966).
6. M. Salyani, 'ASTM STP 1183: Pesticide Formulations and Application Systems,' Vol. 13, American Society for Testing and Materials, Philadelphia, PA, pp. 215–226 (1993).
7. P.C.H. Miller and A.G. Lane, *Aspects Appl. Biol.,* **54**, 45 (1999).
8. H. Ganzlemeier, D. Rautmann, R. Spangenberg, M. Streloke, M. Herrmann, H. Wenzelburger, and H. Walter, 'Studies on the Spray Drift of Plant Protection Products,' Blackwell, Berlin (1995).
9. A.J. Hewitt, 'Methodologies for the Quantification of Spray Deposition and Drift in Field Applications,' ASAE Paper No. 001028, American Society of Agricultural Engineers, Milwaukee, WI (2000).
10. R. Sava, 'Guide to Sampling Air, Water, Soil and Vegetation for Chemical Analysis,' California Department of Pesticide Regulation Handbook EH 94-04, California Department of Pesticide Regulation, Sacramento, CA (1994).
11. M.K. Carroll and J.A. Bourg, *Mosquito News,* **39**, 645 (1979).
12. A.J. Hewitt and T. Megenassa, *Crop Protect.,* **12**, 59 (1993).
13. K.R. May, *J. Sci. Instrum.,* **27**, 128 (1950).
14. P. C. H. Miller, P. J. Walklate, and C. J. Mawer, 'A comparison of spray drift collection techniques,' in "Proceedings of British Crop Protection Council Brighton Conference on Weeds", British Crop Protection Council, Farnham, pp. 669–676 (1989).
15. C.W. Lee, J.D. Parker, D.A.T. Baldrey, and D.H. Molyneux, *Pestic. Appl. News Sheets,* **24**, 404 (1978).
16. S.R. Shewchuk, K. Wallace, and J. Maybank, 'An Evaluation of the Rotorod Sampler for a Forest Herbicide Drift Monitoring Program,' SRC Publications, E-2310-2-E-90, Saskatoon Research Center, Saskatoon, Canada, p. 19 (1991).
17. R.L. Edmonds, *Plant Dis. Rep.,* **56**, 704 (1972).
18. G.A. Matthews, 'Pesticide Application Methods,' Longman, London (1992).
19. B.D. Hill and D.J. Inaba, *J. Econ. Entomol.,* **82**, 974 (1989).
20. C.R. Turner and K.A. Huntington, *J. Agric. Eng. Res.,* **75**, 385 (1970).
21. A.H. Higgins, *J. Econ. Entomol.,* **60**, 280 (1967).
22. R. Sanderson, E.W. Huddleston, J.B. Ross, J.A. Henderson, and E.W. Ferguson, *Trans. ASAE,* **29**, 378 (1986).
23. R.W. Whitney and L.O. Roth, *Trans. ASAE,* **28**, 1749 (1985).

24. H. Zhu, D.L. Reichard, R.D. Fox, and R.D. Brazee, 'Collection Efficiency of Spray Droplets in Turbulent Air,' ASAE Paper No. 941503, Presented at ASAE Symposium, Atlanta, GA (1994).
25. R. Behrens and W.E. Rueschen, *Weed Sci.,* **27**, 486 (1979).
26. N.B. Akesson and D.E. Bayer, ASAE Paper 971075, Presented at American Society of Agricultural Engineers 1997 Annual Meeting, Minneapolis, MN (1997).
27. D.D. Kristmanson, J.J.C. Picot, G.W. van Vliet, and J. Henderson, 'Measuring Foliar Deposits From Aerial Spraying of Pesticides,' Forest Protection Limited and the Natural Sciences and Engineering Research Council of Canada, p. 269 (1989).
28. K.M.S. Sundaram, A. Sundaram, and R. Nott, *Trans. ASAE,* **29**, 382 (1986).
29. W.A. Taylor and G.B. Shaw, *Pestic. Sci.,* **14**, 659 (1983).
30. M. Salyani and J.D. Whitney, *Trans. ASAE,* **33**, 361 (1990).
31. K.R. May and R. Clifford, *Ann. Occup. Hyg.,* **10**, 83 (1967).
32. A.J. Hewitt, D.L. Valcore, and J.E. Bryant, 'Spray Drift Task Force Atomization Droplet Size Spectra Measurements,' in PROC ILASS–Americas 96, San Francisco, CA (1996).
33. A.J. Hewitt, D.R. Johnson, J.D. Fish, C.G. Hermansky, and D.L. Valcore, *J. Environ. Toxicol. Chem.,* **21**, 648 (2002).

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Field methods for performing farm worker exposure and re-entry studies

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1 Introduction

Farm worker exposure to pesticides has been studied extensively over the past 30 years.[1–6] This scientific discipline has evolved from the days when respiratory exposure of farm workers was measured using gauze dosimeters placed inside respirators to collect airborne pesticide residues to very sophisticated air sampling devices and remarkable dosimeter devices to measure dermal exposure to farm workers.[4]

Measurement of respiratory exposure to farm workers has been performed for many years. Early on, researchers described the use of a respirator fitted with filter disks to measure respiratory exposure of farm workers to parathion. These devices were difficult to use since tight-fitting respirators were hard to find, and the respirators themselves created difficulties for the worker in breathing while participating in the study. Furthermore, the filter pad was not a good substitute for a safety canister when handling more toxic pesticides. The most commonly used device for measuring respiratory exposure to farm workers is a personal air sampling device consisting of an adsorbent air tube attached to the collar of the worker, which in turn is attached to an air pump. The air pump is then attached in some manner to the body of the worker. Some of the more recent developments in methods to measure respiratory exposure involve the use of an air sampling train, which consists of an adsorbent air sampling tube attached in series to a Gelman-type filter. This sampling train is then attached to a personal air-monitoring pump. These types of air sampling devices will be discussed in detail later.

Among the first dermal dosimeters used in exposure research were 4×4-in cellulose or gauze patches which were pinned to the outer and inner surfaces of clothing or vests which farm workers would wear during the application or re-entry phase of the study. These patches were easy to manufacture and when pinned to the shirt or pants of the worker made for an easily used dosimeter pad. The major advantage to the use of the patch to estimate worker exposure was this method's ability to differentiate the relative contributions of pesticide residues to different parts of the worker's body. This sampling technique in turn could lead to recommendations (i.e., the use of

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gloves or aprons) to mitigate exposure of the worker. Another advantage of the patch method was that cellulose or gauze patches could be placed under the work clothes to determine how much of the active ingredient penetrated through the clothing, giving an estimate of the actual skin exposure and addressing the value of using long-sleeve shirts and pants during the handling of pesticides.

Based on the patch method to assess worker or re-entry exposure, researchers have developed a database, which may be used to estimate exposure. Each patch from an individual in a study can be entered into the database separately, the residue data from patches from various body areas can be summed to yield a whole-body exposure number, and the data may be sorted as to worker tasks, equipment used, protective clothing worn, formulation types and other parameters. This is the basis for the currently used Pesticide Handlers Data Base (PHED), which was developed through a joint effort in the 1980s of CropLife America [formerly known as American Crop Protection Association (ACPA) and National Agricultural Chemicals Association (NACA)], the Environmental Protection Agency (EPA) and Health Canada.[7–9] The PHED is discussed in detail in another article in this book.

At best, the use of patches yielded a crude measure of the outer or inner dermal exposure for the active ingredient in question. The disadvantage of the use of the cellulose patch or gauze patch was that the patches intercepted varying amounts of the product being used. One patch, for example, on the shoulder may have intercepted very little of the chemical whereas a leg patch may have intercepted as much as a drop of the product. This disparity in the amount of test substance on the patches on different parts of the body could lead to both underestimation and overestimation of the exposure to the farm worker. This inherent problem with the patch method was magnified when the area of the patch containing the pesticide residue was extrapolated to the area of the body that the patch represented. As a result, when the exposure to all the areas of the body was finally added up, the total exposure would be grossly over or underestimated depending on the amount of the active ingredient on a given patch. When one looked at various individuals from the farm community using this technique, the variability in total exposure from one individual to the next would range over more than two orders of magnitude.

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The use of the patch method, although yielding some good data about exposure over the years, started to fade quickly when whole-body dosimeter methodology was introduced. The whole-body dosimetry method gave more precise whole-body exposure values from one worker to another since the whole garment was extracted and analyzed, and one did not have to extrapolate exposure to large areas of the body.[10,11]

The purpose of this article is to present a detailed description of the current field methods for collection of samples while measuring exposure of pesticides to farm workers. These current field methods encompass detailed descriptions of the methods for measuring respiratory and also dermal exposure for workers who handle the pesticide products directly (mixer–loaders and applicators) and for re-entry workers who are exposed to pesticide dislodgeable residues when re-entering treated crops.

2 Current field methods for measuring mixer–loader and re-entry worker exposure to pesticide residues

2.1 Study design and protocol preparation

All worker exposure and re-entry studies should be well thought out and planned in great detail prior to moving the study into the field. Attention to planning and detail are the key to performing a successful worker exposure or re-entry exposure study. No matter whether one is working under strict Good Laboratory Practice (GLP) standards as required by the EPA for scientific studies supporting the registration of all pesticides or working under academic standards where good science is the key element, good planning of the field portions and analytical portions of a study is essential. For the purposes of this article, planning of the field portion of the worker exposure/re-entry study will be of primary importance. The planning of the analytical portion of the study will not be presented in any great detail except where the field and analytical portions of the study interface.

The protocol for a study is one of the most important aspects of the worker exposure/re-entry study. The protocol should contain the design of the study in great detail and should lead the researcher through a logical sequence of steps that, if followed, will allow the scientist to accomplish the research in a way that the study could be reproduced in the field by other interested parties. Since GLP standards are followed in most industry-related worker exposure/re-entry studies, the author recommends a list of the necessary components of the protocol that will inherently produce the best scientific design of the study which would be adequate for industry, government, or academic research projects. Such a list of necessary protocol components can be found in Section 160.120 of the EPA GLP requirements (40 CFR Part 160 of the *Federal Register*, August 17, 1989).[12]

Preparing a detailed protocol according to GLP standards may be useful for researchers planning a worker exposure or re-entry study, even though the study may never be submitted to the EPA to support the registration of a pesticide product. Having a detailed protocol helps to solidify one's plans for the study, and assists in the documentation phase of the study. This documentation phase will be addressed in some detail later.

When planning the details of the field and analytical methods for a worker exposure or re-entry study that may eventually be submitted to support the registration of a pesticide product, one should consult the current EPA guidelines for such studies. The guidelines have been published by EPA under 'Occupational and Residential Exposure Test Guidelines OPPTS 875',[13–20] and may also be useful in an academic setting even though the study may never be submitted to the EPA to support the registration of a product. The EPA guidelines are really 'guidelines' and should not be used in such a way as to stifle creativity when developing plans, protocols, and scientific techniques or experiments to assess worker exposure to pesticides.

2.2 Site selection

One of the most important aspects of designing a successful worker exposure or re-entry study is the selection of the agricultural site at which to perform the study. One must first define the growing region in which the pesticide is to be used to the widest extent possible. This is important since this region would have the most farms, farmers, commercial applicators, or re-entry workers which would have work habits, equipment, and land, which would best represent the use of the product.

One of the first items of importance when attempting to select an agricultural site at which to perform a worker exposure/re-entry study is to review the label on the product. Such a review will reveal the areas where the product is to be used and the conditions under which the product is to be applied. One should look for whether a product is to be applied by ground or air and if there are any restrictions as to the locations where the product is to be used. One must also pay attention to label directions concerning types of soil where the product is to be used since this could affect rates of use.

The location of the study may be secured through communication with local farmers, agricultural extension agents, agricultural trade associations, local growers, local pesticide dealers, or state regulatory persons. The United States Department of Agriculture (USDA) has an extensive web site, which offers the names, addresses, and telephone numbers of many agricultural extension agents throughout the United States who may be helpful in locating the appropriate place to perform a worker exposure/re-entry study. If the research is to be done with individual farmers on their farms, local agricultural extension agents should be contacted who can help direct the researcher to farmers who have assisted in agricultural research projects in the past and will generally provide for a helpful environment in which to do the field research. If the research is to be performed with commercial applicators or commercial re-entry field workers, the researcher may want to contact a local grower or commercial pesticide supply facility to obtain the volunteers for the study and also the equipment and land on which to perform the study. In any event, communication with parties who may assist with the selection of the field site is extremely important and should be completed well before the protocol is completed in order to describe the location of the study in the protocol.

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When selecting a site to perform applicator/re-entry exposure research, the site must be typical for the crop to be treated and typical for the spray equipment to be used. Commercial sites where crops are grown are always good to use because they offer the most typical agricultural practices where pesticides may come into play and the most typical and well cared for equipment, which may be used for the application. These commercial sites can have disadvantages in that the researcher may not be able to control all parameters including the available labor force when the test day arrives. Further, the researcher may not be able to control interfering pesticide applications to site, which are made on or near the test day near to the test site as a result of routine maintenance practices carried out by the commercial facility. In any event, thorough communication is essential between the researcher and the appropriate authorities at the test site in order to overcome such disadvantages.

The other type of site, which is less commonly used for worker exposure and/or re-entry studies, may be found on a research farm. These research farm facilities may

have large plots of land with the appropriate crop for the study. If the equipment to be used to make the pesticide application is typical of commercial equipment, which may exist on a farm site, then such a site affords an ideal location to perform such a study. However, in many instances, the research farm plot may be smaller than is typical for commercial practices, or the application equipment may be more for making custom applications rather than for typical commercial applications. The researcher should not trade the more comfortable study location of a research farm with atypical land and equipment for the more rigorous typical commercial or real farm situation. This is because the best scientific strategy in planning such studies should allow for typical agricultural practices with typical application equipment as a central feature. While more experimental control may be possible when working on a research farm, worker/re-entry exposure research in itself is inherently wrought with experimental variability, and the amount of 'control' over the study gained by working in a more controlled environment (i.e., the research farm) is minimal. Such practices may actually inhibit one from coming up with a 'typical' exposure assessment for the farm workers.

When planning a worker exposure/re-entry study, the number of sites selected should reflect the use pattern of the product. Usually three different sites, each in a different geographical location, are recommended to ensure good representation of the areas when the product has wide use. The different geographical locations offer the researcher diversity in equipment used to treat the crops as well as diversity in the work habits of the individuals in the study.

2.3 Setting up a field laboratory and auxiliary equipment

2.3.1 Locating the field laboratory

The field laboratory set up by the field research group is a key element to completing successful worker exposure/re-entry research. The field laboratory may be set up in close proximity to the treated field, but should be located at a reasonable distance from the treated area to avoid cross-contamination of field samples and field controls.

The field laboratory must have running water, lavatory facilities, and electricity to facilitate hygiene of the Field Scientist crew and also to facilitate the use of air pump battery chargers and office equipment such as portable copiers which may be of value during the course of the field research.

2.3.2 Supplying the field laboratory

The supplies that are to be used for performing the worker exposure/re-entry study should be moved to the field laboratory well ahead of the first test day. These supplies may generally consist of aluminum foil for wrapping dosimeter samples, sealable bags for containing samples, jars for liquid samples such as hand washes and test substance retainer samples, labels for samples, clear tape for labels, tape dispensers, air sampling tubes, air sampling pumps, calibration equipment for pumps, cooler and shipping boxes, freezers, dry-ice, wet or blue ice, fold-up tables for holding supplies in the field laboratory, and many other items needed to accomplish the

study. Many of these supplies may be purchased at local supply stores or through research supply companies. Personal computers with Internet software, laptop notebooks, fax machines, and portable copiers should also be moved to the field laboratory to provide the field research staff with the most modern communication and office technology.

2.3.3 Use of portable weather stations and hand-held weather equipment at the field laboratory

Portable weather stations are useful to have available at the field laboratory for acquiring weather data during the course of the worker exposure/re-entry study. There are a variety of portable weather stations available from a variety of suppliers. Weather data to be collected are rainfall, wind direction, wind speed, air temperature, and relative humidity. These electronic weather stations will record the necessary weather parameters on a routine basis. The data are stored and can be transferred to a laptop computer or disk as desired by the Field Scientist. Such portable electronic weather stations are useful during the course of the dislodgeable residue portion of a worker re-entry study when dislodgeable residue samples are taken from remote test sites over the course of a 30-day period.

In addition, there are hand-held psychrometers for measuring air temperature and relative humidity available, and also hand-held wind meters, which measure wind speed. When using such hand-held equipment, readings should be taken each hour during the course of the day when worker exposure volunteers or re-entry worker volunteers are performing the test.

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2.3.4 Calibration of field equipment at the test site and field laboratory

All equipment to be used at the field site should be calibrated at or near the field laboratory or field site prior to the application of the test substance (pesticide product). Most weather equipment will have been calibrated at the manufacturer and can be checked for functionality prior to the worker exposure/re-entry test by comparing weather readings from the nearest airport or National Oceanic and Atmospheric Administration (NOAA) weather reporting station with the weather readings compiled by the portable weather station. Hand-held weather instruments could also be checked with current weather reading from local airports or NOAA facilities.

Air sampling pumps should be calibrated and the planned flow rate should be set prior to using them in the study. The calibration of these air pumps should be accomplished with an air sorbent tube in place, which is similar to the type to be used in the study. Records on the calibration of the air pumps should be kept in the field research notebook for the study.

Air sampling pumps should be checked for flow rate after the completion of the exposure replicate. Some researchers use the air tubes in the test to check the flow rate of the pump with which it is associated and others use a representative air tube to check the air pumps post-exposure. It is not clear which method is best. However, using the test air tubes to check the flow rates post-exposure has some disadvantages in that the tubes may be cross-contaminated from too much handling, or samples may be mixed up for the same reason.

Equipment to be used for the application of the test product should be calibrated just prior to the application date. Such equipment should not be used for any other application prior to the test application after the equipment has been calibrated.

1. Ground boom application equipment should be calibrated as follows:

   (a) The application rig should be checked to make sure all pumps and hoses are intact and not leaking.
   (b) All nozzles should be checked to ensure that they are the correct nozzles called for in the protocol or plans for the study.
   (c) The tank of the equipment should be filled with water, and the sprayer should be activated for about 30 s to ensure all nozzles are visibly working, then the sprayer should be turned off. The sprayer should be turned on again for about 30 s, then graduated cylinders or other marked collection cups should be placed under the nozzles, and the water should be collected for about 30 s. The amount of the water in the collection devices should be recorded. If the output of any nozzle is more or less than 10% of the average output of all the nozzles, the nozzle(s) in question may have to be cleaned, repaired, or replaced. This procedure should be repeated until the output of each nozzle on the rig has been checked in triplicate, and all are within the 10% tolerance.
   (d) The ground boom rig should now be timed three times along a 100-ft distance, and the times should be recorded. The average time of the spraying and the average nozzle output volumes are to be used to calculate the gallons per acre of carrier (usually water) to be used when making the application of the test substance and is referred to as the calibrated speed of the spray rig.
   (e) The pressure of the pumps on the spray rig and parameters such as the gear number used to accomplish the average speed of the rig should be recorded in order that the tractor rig can be set up at the time of application to reproduce the application parameters established during calibration of the spray rig.

2. Aerial application equipment should be calibrated as follows:

   (a) All nozzles should be checked for functionality and to determine whether the nozzles to be used are appropriate to provide the correct application. One should check all hose connections and pumps to ensure that these important elements are fully functional.
   (b) A 1500–2000-ft strip of surface along a runway is marked off with bright colored tape at both ends. A flagger is positioned at one end of the strip while a technician with a stopwatch stands at the other end.
   (c) The airplane (spray equipment) is positioned on a flat surface near the mixing area, and the position of the wheels of the plane is marked with bright-colored tape.
   (d) A pre-calibrated water meter is hooked into the water hose used to fill the spray tank of the airplane, and the amount of water used to fill the tank is recorded. The water meter is checked using a 5-gal bucket (which has also been checked for volume accuracy and marked at 5 gal).
   (e) A commonly used dye (optional) is mixed in the spray tank with a known amount of water (measured by the water meter), and the level to which the water rises

in the spray tank is marked. A yellow or white strip of tape should be stretched across the runway at the place where the timer is standing.

(f) The airplane then flies over the marked runway and begins to spray the dye at the first marker where the flagger is standing and stops spraying at the mark where the timer is standing.

(g) The flagger drops the flag when the airplane crosses at a point directly in front of him/her so that the timer positioned down the runway can start timing the application. The timer stops the stopwatch when the plane passes directly in front of the timer. The recorded time is the time to spray the 2000-ft swath from which air speed can be calculated.

(h) The width of the spray swath is then marked and measured by observing where the dye has been deposited on the tape, which was stretched across the runway.

(i) The airplane then returns to the loading area and parks with the wheels on the tape used to mark the first position of the plane.

(j) The water meter is used to determine how much water (carrier) was used to spray the measured swath width. This is done by first setting the water meter to 0, filling the spray tank up to the original marked level, recording the amount of water to fill the tank to that position, and then calculating the amount of water used to spray the runway. The information gathered at this point can be used to calculate the anticipated application rate and carrier volume to be used in the actual calculation.

(k) If the calibrated rate of application is not what is desired, nozzles on the airplane boom may have to be replaced or adjusted.

(l) The timer is also responsible for recording the air temperature, wind direction, and wind speed at the runway site just prior to the airplane making the calibration spray run.

(m) After all calculations have been performed and the appropriate anticipated rate of application has been achieved, parameters used to achieve the desired application rate should be recorded in order that these parameters can be reproduced at the time of application of the pesticide.

(n) The procedure for calibrating an airplane rig should be repeated in triplicate.

3. Air blast applicators should be calibrated as follows:

(a) All nozzles should be checked for functionality and to determine if the nozzles to be used are appropriate to provide the correct application. All hose connections and pumps should be checked to ensure that these important components are fully functional.

(b) A 300-ft long section of trees to be sprayed is measured using a measuring wheel and marked with clearly visible flags.

(c) The gears on the spray rig are set to the desired speed, and the speed of the air blast sprayer is checked by timing its movement over a distance of 100 ft three times.

(d) The air blast rig is parked in a level spot and the location of the wheels is marked. The spray tank is filled with a known amount of water. One option is to use a water meter to measure the amount of water placed in the tank. Another option is to use volume-sighting marks on the tank that have been previously verified. The amount of water added is recorded.

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(e) The 300-ft length of trees is sprayed at the desired rig speed and nozzle output, while the spray operation is timed from the outer edge of the first trees to be sprayed to the outer edge of the last tree to be sprayed. The rig is then brought back to the same spot where the fill-up took place. The amount of water left in the spray tank is then measured to determine the amount of the carrier sprayed.
(f) The parameters recorded during the calibration of the air blast sprayer can be used to calculate anticipated application rates and carrier volumes for the application of the test substance.
(g) The calibration procedure for the air blast sprayer should be repeated in triplicate. Changes in nozzle output or changes in the speed gears of the air blast sprayer can be adjusted until the appropriate application rate is achieved.

2.3.5 Receipt and storage of test substances and reference substances

(1) Test substances

The test substance is generally defined as the formulated pesticide product which is being applied to a crop or field and for which worker exposure is being assessed.

The test substance may be acquired in a number of ways. One common method is to purchase the product from a chemical dealer. This method of acquiring the test substance for a worker exposure or re-entry study allows one to proceed rapidly with the execution of the field portion of the study without the serious delays encountered while waiting for a test substance to be manufactured, assayed, and shipped to the site. Although having a test substance formulation which has been assayed under GLP standards and for which a certificate of analysis has been shipped to the test site is desirable, there are several instances when this is not practical. For example, one may encounter a worker exposure study where the pesticide to be evaluated is sold only in mini-bulk or even large bulk quantities holding as much as 2000 gal. In this case, duplicate 5–10-g or 5–10-mL retainer samples should be taken from the bulk or mini-bulk tanks for assay after the worker exposure study is in progress.

In many instances under GLP, the test substance may be manufactured and assayed for the specific purpose of use in a worker exposure or re-entry study. This is normally done when the amounts of the product to be used are relatively small, and the product is not sold in bulk. In this case, a certificate of analysis should be obtained prior to the initiation of the field portion of the study. In this case, the manufacturer should retain some of the material for periodic reassay to meet GLP requirements.

The receipt of the test substance should be documented upon arrival at the test site. The name of the product, manufacturer, active ingredient concentration, expiration date, storage location, storage requirements, lot or batch number, the amount received, the condition at receipt, and whether the material is an emulsifiable concentrate (EC), flowable, powder or otherwise should be noted in the research notebook. In addition, one should note the purchase date, the shipment date, and the carrier of the product.

In some instances when dealing with test substance products sold in bulk or mini-bulk, one must obtain shipping papers or bills of lading from a chemical dealer to document the lot number and origin of the test material and how the test material reached the field site.

The test substance should be stored in a locked facility at or near the test site. Most pesticide products are manufactured to withstand extreme temperatures, therefore most test substances used for re-entry and worker exposure studies may be stored under ambient conditions. One should read the label of the product carefully to discern if there are any extraordinary storage conditions required for the pesticide product. Temperatures at the test substance storage location should be monitored daily using a max/min thermometer or similar device that can record daily fluctuations of temperatures.

(2) Reference substances

A reference substance can be either the formulated test substance suspended in water or the technical or analytical grade active ingredient of the test substance dissolved in a solvent. The reference substance is normally used to fortify field matrices to develop information on the field storage stability of the active ingredient. Reference substances should be prepared at the analytical facility where the matrix samples are to be analyzed. Methods to prepare reference substances for field use will be discussed later.

Upon receipt of the reference substance at the field site, one must document the following: name and source of reference substance; the date of shipment and receipt; the condition at receipt (e.g., seal broken or not, reference substance frozen, cold, or ambient when received); the lot number; expiration date; total amount received; and storage requirements. A log should be kept in the field which documents the usage of the reference substance. The lot or reference number, date used, how much used, how much left, and the disposition of the reference substance should be recorded.

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Reference substances should be stored under appropriate conditions in temperature-regulated rooms, refrigerators, or freezers. The temperatures of the storage device should be monitored with max/min thermometers or other temperature-recording devices on a daily basis. Having a small generator or dry-ice available may be necessary in case there is a power outage, and the storage device fails.

Acquiring freezer or frozen storage capacity at a field site is sometimes a major logistical problem. Obtaining and keeping samples on dry-ice in large coolers at the site is one option, but this can become expensive very fast. One alternative is to buy chest freezers. These freezers can be bought used or new and are not expensive when compared with the availability, cost, and transport of dry-ice to be used for long-term storage of samples in the field. Another option is to have a freezer truck at the test site to store the samples and to move them directly to the analytical laboratory.

2.4 Acquiring consent from study participants

2.4.1 Preparing a consent form

The consent form is a document to be used to acquire the consent of worker volunteers to become a part of a worker exposure study or re-entry exposure study and is an integral part of ensuring the ethical integrity of the research project. This form is normally in English but should be translated into Spanish or any other language

which would accommodate the potential volunteers in the study. The form should contain the title of the study, the protocol number, and reference to the sponsor and any subcontractors hired by the sponsor to perform the study. The form should state the purpose of the study and why the worker is being asked to participate. The procedures which the volunteer will be performing during the study should be explained in some detail in the form; however, the language should be straightforward and simple so as to be understood in lay terms. The form should also have an area to record the height, weight, age, work experience, and general health of the individual. In addition, the form should discuss the expected risks and discomforts to the worker when participating in the study and the costs and benefits to the worker for participation. The form should also contain information which gives the volunteer confidence that his/her normal work activities or employment will not be jeopardized by his/her involvement or noninvolvement in the study. The form should also state that the study participant could withdraw from the study at any time during the course of the study without any penalty to the volunteer. The consent form should address the manner in which the volunteer's name is to be held in confidence and methods to obtain medical attention if injured as a result of performing in the study. There should be a section in the consent form listing the names and phone numbers of appropriate individuals to call to ask questions about the study and to ask for follow up information on the study. Finally, a section should be included which allows the volunteer as well as the person obtaining the consent to sign and date the form.

2.4.2 Review of the consent form by ethical review boards

Most consent forms that have been developed have at some time been reviewed by standing ethical review boards, usually associated with a university. The format of the consent form described above has been reviewed over time by more than one ethical review board and would be acceptable in most States for the purpose of acquiring the consent of potential volunteers in a worker exposure or re-entry study. If performing worker exposure studies in California, the researcher is advised to contact the State regulatory agencies and submit the draft consent form and study protocol to the State-appointed ethical review board for review and approval prior to initiation of the field phase of the study. Of course, if the researcher has any doubt about the acceptability of the proposed consent form, he/she should contact the appropriate state agencies where they plan to perform the study.

2.4.3 Meeting and selecting the volunteers for a worker exposure/re-entry study

Volunteers for a worker exposure or re-entry study should be selected with care and with confidentiality. The volunteer's privacy and also respect for the volunteer should be kept in mind during the field phase of the study. With all worker exposure/re-entry studies, the worker volunteer is the key to a successful study.

Workers are normally chosen for their skills and experience in carrying out the required tasks for the field portion of the study. For example, if one were doing research on exposure of custom applicators during treatment of corn in the Midwest USA, the researcher would probably contact an agricultural extension agent in the part

of the country where the study is planned. This agent can provide information on the commercial application facilities in the area in question. Next, the researcher would contact the commercial application facility and set up a meeting with the foreman or manager to discuss the study and meet potential volunteers. At the first meeting of the volunteers and researcher, the requirements of the study should be outlined, and information about how to contact the volunteers in the future should be obtained from the foreman and/or the potential volunteers directly.

Communication with the potential volunteers prior to the study is essential. Prior to the initiation of the field phase of the study, a meeting should be held with the potential volunteers to explain the details of the procedures of the study, present the consent form, and inform the potential volunteers of the risks and benefits (including any monetary compensation to the worker) of participating in the study. At this meeting, consent in writing should be obtained from the worker. If there is any language barrier between the researcher and the potential volunteers, an interpreter should be present at the meeting.

Finally, at this meeting, the volunteers should be instructed on when and where to go to the test site or field laboratory in order to start the study and should be reminded to bathe or shower on the morning of the test prior to attending the test site.

2.5 Execution of the field portion of the worker exposure/re-entry study

2.5.1 Arrival of the study participants at the test site

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The study participants (volunteers) should arrive at the field laboratory well before the daily work activities are to commence. The study participants should be directed to sit near the dressing area on a seat covered with a fresh plastic bag or tarp. The volunteers are usually instructed not to move from their seats or wander off around the test site. Control of the movements of the study participant is crucial at this point since the worker could encounter contamination and acquire some extraneous exposure not planned for the study.

While the participants are waiting to be dressed for the study, a Field Scientist can obtain vital information on the test subject such as weight, height, age, and work experience, which may not have been obtained previously.

2.5.2 Preparing the study participants for the field phase of the study

(1) Processing the volunteers prior to dressing in dosimeters

Coordinating the activities of the test subjects prior to starting an application scenario with the workers requires considerable preparation and expertise on the part of the group that is performing the study. The test subjects are usually brought to the field laboratory or an area equipped with appropriate supplies to perform such activities as face wipes and hand washes prior to initiation of the dressing procedure. Such hand washes and face wipes prior to the initiation of the field phase of the study allow for background checks of contamination to be performed. These background samples can be of value in interpretation of the hand wash and face wipe data gathered during the

course of exposure, since knowing the extent of hand and face contamination prior to beginning an exposure scenario is necessary.

(2) Dressing of the test subject in dosimeters

After the pre-exposure face wipes and hand washes have been performed, the volunteers should be directed into a private dressing area to dress them in the dosimeters that they are to wear during the exposure period. The dressing area is usually a room outfitted to provide maximum privacy for the volunteers. Windows are usually covered with curtains, and doors remain closed during the dressing procedure. A chair covered with aluminum foil or fresh plastic bag can be provided to the volunteer for sitting during the dressing procedure. Replacing this foil or bag between dressing of volunteers allows prevention of cross-contamination of the active ingredient between volunteers. A Field Scientist is usually present during the dressing procedure to supervise the volunteer and to provide surgical gloves to the volunteer at critical times during dressing.

The volunteer will first sit in the chair, and the Field Scientist will provide him/her with a pair of fresh, never-used surgical gloves. The volunteer will then undress, placing his/her shoes, socks, pants and shirt in a box provided by the Field Scientist. The volunteer should have been instructed at a prior time to wear an undergarment which would provide some privacy during undressing such as gym shorts or a swimsuit. On occasion, a tee shirt and brief may be part of the dosimetry equipment to be used in the study. In this case it will not be necessary for the volunteer to wear gym shorts or a swimsuit under his/her clothes.

After the volunteer has completed undressing, the volunteer will change to a fresh pair of surgical gloves provided by the Field Scientist. At this time, the Field Scientist will hand the volunteer an inner dosimeter to put on. The inner dosimeter may be either a whole-body dosimeter such as long underwear, or a tee shirt and brief type underwear, which are sometimes used as an inner body dosimeter. After the volunteer has donned the dosimeter, the Field Scientist will provide whatever necessary outer clothing for the volunteer to put on. Some study designs require new, unused outer clothing to prevent any cross-contamination of the volunteer's dosimeters. These outer garments may be either seasonal clothing worn normally by the worker or may be actual outer whole-body dosimeters which will be analyzed for residues of the active ingredient at the termination of the field portion of the study. Such outer whole-body dosimeters may be purchased at local retail stores or may be bought wholesale from various suppliers.

After donning the outer dosimeter or clothing, the volunteer will then put on his/her socks and shoes. Usually clean, unworn socks are provided to avoid cross-contamination of the inner dosimeter with the socks from prior workdays. New shoes are also provided to the volunteer, again to avoid cross-contamination issues. On occasion, regular pre-worn shoes and socks may be used for the volunteer if cross-contamination of samples is not an issue.

At this time, the volunteer exits the dressing room and is provided with a clean air monitoring pump which is hooked to the belt or outer dosimeter of the volunteer. The volunteer is now ready to move to the test site. If multiple volunteers are to be prepped and dressed, volunteers who are ready to go to the field site are set back in the chairs to wait for all volunteers to be dressed.

(3) Moving the volunteer test subjects to the field site

After the volunteer is dressed and outfitted with appropriate gear for the study, he/she may have to walk only a short distance to the field site or may need to be transported to the field site via a vehicle. When transporting the volunteer(s) by a vehicle, the seats of the vehicle should be covered with plastic sheeting. The volunteers should be escorted into the vehicle and asked to keep their hands off surfaces in the vehicle. The volunteers are then transported to the field site. The plastic seat covers need not be changed until after the volunteers have been transported back to the dressing area for post-exposure processing. The plastic seat covers should be changed before a new set of volunteers enters the vehicle.

(4) Processing the test subjects post-exposure

After the exposure period is completed, the volunteer is returned to the dressing area, where the air pump is removed and a face wipe and hand wash are performed. The volunteer is then taken into the private dressing area set aside for undressing the test subjects. This is a separate room from the room where the volunteer was dressed but is outfitted similarly to the dressing room with a chair covered with fresh plastic or unused aluminum foil. Additional aluminum foil is usually placed on the floor and changed as needed to ensure that the volunteer's shoes do not cross-contaminate his/her socks as the undressing proceeds. The volunteer is now handed a pair of surgical gloves by the Field Scientist and asked to remove his/her shoes. When the shoes are off, the volunteer's surgical gloves are changed, and he/she is instructed to take off the socks. The gloves of the volunteer are changed again, and he/she removes the outer dosimeter. This procedure is repeated with the inner dosimeter, being careful to use fresh surgical gloves with each new layer or garment in order to avoid cross-contamination of the inner dosimeter with the outer clothing or socks. At this point, the volunteer puts back on his/her street clothes and is ready to exit the dressing station.

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2.5.3 Field techniques for performing worker exposure/re-entry research: direct measurement of exposure

(1) Whole-body dosimetry

Both inner and outer whole-body dosimeters are common tools to measure successfully dermal exposure to pesticide workers and are employed in a variety of ways in mixer-loader/applicator or re-entry studies.

Selection of the type of whole-body dosimeter is important. Inner whole-body dosimeters are usually white, 100% cotton, long underwear purchased from a variety of clothing outlets and stores. One- or two-piece inner whole-body dosimeters are common. Outer whole-body dosimeters can range from hand-made cotton coveralls to shirts and pants bought directly off the shelf at local retail stores. Outer whole-body dosimeters can also be purchased from wholesale clothing outlets. Outer whole-body dosimeters may be any color and may also be 100% cotton or mixed materials, depending on the purpose for which the outer whole-body dosimeter is to be used. For example, one may want to use a coverall as an outer whole-body dosimeter. This would be acceptable even if the coverall were not white and not 100% cotton provided that the fabric did not contain interfering analytical components.

Pre-exposure processing and preparation of the inner and outer whole-body dosimeters for use in the field should be considered. The analytical laboratory should determine if the fabric of the dosimeter of choice contains any analytical interference, which may be a problem in subsequent analysis of the fabric. If such analytical interferences are present in the fabric of the dosimeter, they may be reduced by pre-washing the dosimeter material prior to use in the field. The dosimeter is usually pre-washed (sometimes more than once) and rinsed several times prior to thorough drying. The washing detergent of choice should be as free as possible from additive brighteners and other chemicals, which may cause analytical interferences.

Storage of the whole-body dosimeters prior to the initiation of the field phase of the study should be considered. Whole-body dosimeters are usually stored in sealable plastic bags prior to use in the study. If the dosimeters come from the manufacturer in enclosed bags, they should be left in the bags, and the bags should be placed inside another sealed bag for storage prior to the study. If the dosimeters are pre-washed, they should be immediately placed in plastic sealable bags after drying and cooling to room temperature.

Whole-body dosimeters are processed post-exposure as follows. The whole-body dosimeter is laid on a table covered with fresh aluminum foil and is sectioned into various pieces using a solvent-cleaned pair of scissors. The whole-body dosimeter is usually cut just at the knees to provide two lower leg sections, at the waist to provide an upper leg section, at the elbow to provide two lower arm sections, at the edge of each shoulder to provide two upper arm sections, and across the shoulders and down each side of the chest area to provide a front torso and back torso sample. The two-dosimeter sections from symmetrical parts of the body are combined to form one sample and wrapped in aluminum foil prior to storage. The upper leg, front torso, and back torso pieces are kept separate, and each is wrapped in aluminum foil prior to storage.

An alternative method for cutting the whole-body dosimeter is to hang the garment on a line by the shoulders and cut the garment while hanging. This technique will avoid dislodgement of the pesticide residues, which may occur during contact of the garment and the aluminum foil on the table.

(2) Patch techniques for measuring body exposure to test subjects

Patches have been used for many years to measure dermal exposure to mixer–loaders/applicators and re-entry workers. The patches are usually distributed over the body of the worker and pinned or clipped to designated areas of the clothing, which the worker wears. Patches may be placed on top of the clothes to measure outer clothing exposure or underneath the clothing to measure how much of the pesticide would potentially move through the outer clothing to the skin of the individual. Alternatively, patches have been covered with cloth material and worn on the outer clothing to mimic a patch, which may be worn under the clothing. This alternative technique has fallen out of favor with worker exposure scientists as of late.

Patches are designed a number of ways. Most patches are of size 4×4 in. The patches may be made of several layers of surgical gauze or cellulose. The patch should have a backing composed of a lightweight, hard material covered with aluminum foil. The gauze or cellulose can then be secured to the backing itself using Velcro or safety pins. This backing will prevent the cellulose or gauze part of the patch from serious

degradation during the course of the study replicate if the patch somehow becomes wet.

Patches, once manufactured, should be stored in dry, sealable bags until needed in the field. This will prevent contamination of the patch and degradation of the patch during transport to the field site.

Patches are placed on the test subject at designated locations. According to Durham and Wolfe,[21] one patch should be placed on the top of each shoulder; one on the upper chest near the jugular notch; one on the back of the neck at the edge of the collar; one on each upper front leg (thigh area); one on each lower front leg (just below the knees); and one on the back of each forearm. Patches may also be placed on the front and back of a hat or cap to measure exposure to the face and neck area. When collecting the patch samples, one may prefer to combine the two shoulder patches as one sample; both lower arm forearm samples as one sample; both lower front leg samples as one sample; and both upper front leg samples as one sample, in order to examine the entire area of the body that the two patches represent.

Patches are removed from the test subject immediately after the study replicate is completed. The Field Scientist should wear disposable surgical gloves to remove the patches and change gloves often to reduce the chance of cross-contamination of samples. Individual patches are wrapped in aluminum foil prior to placing them in a pre-labeled sealable plastic bag. Combined patches are placed exposed face to face, wrapped in aluminum foil, and stored frozen in sealable plastic bags. Patch samples may be placed in dry-ice in a cooler or directly into a freezer for storage.

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(3) Techniques for measuring hand exposure

Hand exposures of mixer–loader/applicator and re-entry workers have been measured using a variety of techniques in the past. The most common methods are using gloves as dosimeters and washing the hands with various solvents post-exposure.

(a) Use of glove dosimeters to measure hand exposure

Cotton gloves have proven to be the best type of glove to use to measure hand exposure to workers. The gloves may be purchased inexpensively from a variety of manufacturers. The ‘pall bearer type white cotton glove’ has been touted as the best glove to use since it is very flexible and allows the worker to carry out most hand-related tasks without interference from the fabric itself. Gloves used for determining hand exposure should be checked for analytical interferences and pre-washed if necessary to achieve a ‘clean’ glove without analytical interferences. Gloves should be stored in clean, plastic, sealable bags or other containers to avoid contamination of the gloves prior to use.

The gloves are placed on the test subject just prior to initiation of testing by a Field Scientist wearing clean surgical gloves. The gloves are removed from the test subject after testing is over by a Field Scientist wearing fresh surgical gloves and placed in a freezer for storage.

Occasionally, hand exposure must be tested while wearing protective gloves. This may be easily accomplished by wearing the protective gloves over the top of the cotton gloves.

(b) Use of hand wash techniques to measure hand exposure

Currently, hand exposure is predominately measured using the hand wash technique. Numerous solvents have been suggested for use in this procedure; however, rubbing alcohol or a mild detergent in water has been the most commonly used.

When using rubbing alcohol, the alcohol is first placed in a plastic bag and the hands are inserted into the bag and rubbed for a short length of time (30 s). The hands are then removed and dried. The use of rubbing alcohol as a hand wash has come under some criticism owing to the potential detrimental effect that the alcohol may have on the skin of the study volunteer.

Lately, the use of rubbing alcohol has given way to the use of a mild detergent dissolved in water. The use of mild detergent has the advantage of being less harsh on the skin and may be similar to the soap and water which the test subject would use for normal hygienic purposes. The test subject's hands are washed in about 250 mL of 0.01% Aerosol OT dissolved in water. The detergent solution is poured over the hands, while the hands are held over a bowl. The test subject then rubs his/her hands for 30 s in the bowl using a rubbing motion that mimics washing one's hands in a sink. Alternatively, the worker may simply immerse his/her hands in the bowl of detergent solution and rub them together for the desired period of time. After 30 s the hands are removed from the solution, the wash is poured into a jar, and a new 250-mL volume of detergent solution (or distilled water) is added to the bowl. The test subject then washes his/her hands again for 30 s, the hands are removed from the solution, and the test subject dries them on a paper towel. The second wash is combined with the first hand wash in a pre-labeled sample jar, and the sample is immediately stored frozen.

(4) Techniques for measuring face/neck exposure

(a) Face/neck wipes

The use of face/neck wipes (commonly called face wipes) is probably the most common method of assessing face and neck exposure. Surgical gauze (several ply thick) or handkerchief material (folded in several layers) is generally used. A small volume [usually 8–10 mL of 0.01% (v/v) of Aerosol OT in water] is usually added to the gauze or handkerchief just prior to wiping the face and neck of the worker. Alternatively, the gauze or handkerchief material may be placed in about 25 mL of the detergent solution, which covers the bottom of a jar, the material removed and the excess detergent squeezed from the face wipe prior to using it to wipe the face and neck of the worker. Usually, the worker's face and neck are wiped in a circular fashion, wiping the face with light pressure for about 15 s (covering all the exposed skin area), and then wiping the neck in a circular fashion for another 15 s. The face wipe sample is then placed in a pre-labeled jar, and the procedure is repeated with a fresh face wipe wetted with the mild detergent solution. The second face wipe is added to the first sample in the jar, and the combined sample is frozen immediately on dry-ice or in a freezer.

(b) Patches used to measure face and neck exposure

In the past, 4×4 in cotton patches (gauze or cellulose) have been used to estimate face and neck exposure. The patches are designed and manufactured as described above and are pinned or attached with Velcro to the back and front of a hat or protective headgear of the worker. When the exposure period is complete, the patches are removed, and

the exposed sides of the patches are placed face to face prior to wrapping them in aluminum foil or placing them in another appropriate pre-labeled container. The samples are then stored frozen until analyzed.

(5) Techniques for measuring foot exposure

The measurement of foot exposure to pesticides is not commonly performed in worker exposure studies. However, the measurement of foot exposure can be accomplished by either using socks as a foot dosimeter or by washing the foot using similar procedures to those used for the face wipe.

(a) The use of socks to measure foot exposure

Foot exposure of workers can be measured by using socks as dosimeters or anklet dosimeters worn under ordinary socks. Usually an ordinary sock or anklet bought at a retail store will suffice for the dosimeter. As with whole-body dosimeters, the sock material should be 100% cotton but may be other materials. Pre-washing the socks or anklets prior to use in the field may be necessary if analytical interferences are found in the sock material. The socks or anklets should be put on the volunteer using procedures similar to those already described in order to avoid cross-contamination of the sock dosimeter.

(b) The use of the foot wash to measure foot exposure

The foot is washed with a 4 × 4 in gauze (8-ply) wetted with 8 mL of 0.01% Aerosol OT solution in water by rubbing the top of the foot using a longitudinal motion over the top of the foot for 30 s. This is followed by rubbing the sides and bottom of the foot including the ankles (to just below the ankle bone) for another 30 s in a circular motion. The gauze pad is then placed in a pre-labeled jar, and the procedure is repeated with a second wet gauze. The two gauze samples are combined as a single sample in the same jar.

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(6) Field fortification of worker exposure matrix samples

(a) Purpose for having field fortification samples

Field fortification (commonly referred to as field spiking) is the procedure used to prepare study sample matrices to which have been added a known amount of the active ingredient of the test product. The purpose for having field fortification samples available in a worker exposure study is to provide some idea of what happens to the test chemical under the exact environmental field conditions which the worker experiences and to determine the field storage stability of the test substance on or in the field matrix materials. Field fortifications do not serve the purpose of making precise decisions about the chemical, which can better be tested in a controlled laboratory environment. The researcher should not assume that a field fortification sample by its nature provides 100% recovery of the active ingredient at all times. For example, a field fortification sample by its very nature may be prone to cross-contamination of the sample from environmental contaminants expected or not expected to be present at the field site.

Such field-fortified matrix samples are absolutely necessary as a part of any worker exposure or re-entry study in order for the behavior of the active ingredient to be

understood while on or in the sample matrix during the course of the field phase of the study. As a result of preparation and analysis of such field-fortified matrix samples, one can discern how the test substance behaves on the various media (matrices) used to define the exposure of the worker. For example, one might ask whether evaporation, photolysis, hydrolysis, or other physicochemical reactions might occur on the surface of or in the matrix when exposed to the environmental conditions of the field. A loss of the active ingredient from a dosimeter or patch worn by the worker could lead to an underestimate of exposure if one did not perform field fortification recovery experiments under the same environmental conditions which the worker experienced during the course of the field study. Field fortification experiments are a major concern when performing worker/re-entry exposure studies and, when performed correctly, they provide a quality control element to the study, which allows for correction of exposure estimates to be used in regulatory risk assessment.

In addition, the use of field fortification samples measures the 'carefulness factor' of the Field Scientist during the field research and allows a Study Director/Manager or distant observer to obtain a quality control estimate on the field portion of the study. For this reason, the field fortification samples are usually meant to be different from laboratory procedural fortifications and are meant to be prepared under field conditions, which are considerably more rigorous than are controlled laboratory conditions. For example, environmental factors such as heat, humidity, wind, human stress, and other human factors such as fatigue to the Field Scientist are an integral part of any field worker exposure/re-entry study. Field fortifications made to matrices under these conditions will test and readily demonstrate the ability of the Field Scientist to perform such a difficult study under trying circumstances.

Field fortifications have also been used to measure the storage stability of the analyte in/on exposure matrices during freezer storage prior to analysis. Although use of field fortification samples for freezer storage stability is not the original purpose intended for field fortification samples, this has become an acceptable practice among scientists who work in this scientific discipline.

(b) Preparation and storage of field fortification matrices prior to the field study

Many types of matrices have been used in the past to measure the field stability of the test substance. Cotton gloves, cellulose patches, face wipe handkerchiefs and/or gauze face wipe matrices, long underwear (inner dosimeters), pants, shirts, coveralls (outer dosimeters), sorbent tubes, urine, and other matrices are common matrices that have been used for this purpose.

The preparation of field fortification matrices requires some planning long before the initiation of the field portion of the study. Gloves, cellulose patches, inner/outer dosimeters, face wipes, air tubes, and other matrices should be prepared in a 'clean room' or area away from any stored test product. In addition, cloth, cellulose, or gauze matrices may have to be extracted in the laboratory or washed in a washing machine prior to use in the field to remove any analytical interferences. After extraction/washing, the matrices should be allowed to cool and dry thoroughly and then be placed in zip-type plastic bags for storage prior to delivery to the field.

After initial processing, the entire whole-body dosimeter can be used for field fortification and treated as one sample or can be cut into pieces as described above. Each

piece of the whole-body dosimeter can equally represent a different field-fortified sample. For example, a front torso piece can be used for fortification at a low rate of active ingredient or at a high rate. The lower leg piece can also be used for low or high field fortification samples since the objective is to determine the behavior of the active ingredient on the material under extremes of environmental field conditions. The whole-body dosimeter or sections of it to be used for field fortifications should be stored as separate samples in pre-labeled sealable plastic bags prior to use in the field. The pre-labeled sealable bags in which the dosimeter section is stored can also act as a receptacle for storing the spiked matrix sample after exposure (weathering) under field conditions.

Maintenance of the integrity of matrices used for field fortification samples is of the utmost importance to the field investigator since cross-contamination of the matrix prior to field fortification could lead to field spike recoveries for matrices of a questionable nature. The matrices to be used for field fortification samples must be maintained in a pristine state prior to use in the field. Inadvertent contamination of the field fortification matrices will invalidate any field fortification samples which are prepared. Extreme care must be taken to ensure that these matrices stay free of any residue of the test substance.

Biological fluids such as urine should be collected from individuals with no known exposure to the active ingredient of the test product. These samples should be collected in tarred 2–4-L jars or vessels with nonleak lids and stored in a freezer or in a cooler of dry-ice away from any test product or treated samples until used for field fortification.

(c) How many field fortification samples should be provided during the course of a worker exposure/re-entry field study?

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This is one of the most frequently asked questions by researchers who plan to perform such studies and there is no one set answer. Generally, the number of replicates of field fortifications depends on the size of the sampling regime put in place for the test subjects. For example, the EPA usually requires 15 distinct replicates for mixer–loaders and 15 distinct replicates for pesticide applicators or 15 replicates for mixer–loaders/applicators if the job function of the workers is that of a combined mixer–loader and applicator. It is a generally accepted practice to perform a number of field fortification sets throughout the course of the field phase of the worker exposure or re-entry study depending on how many actual exposure days that the workers are monitored. A field fortification set may consist of three replicate fortifications at each of two levels of the active ingredient under test for each matrix tested. For example, if a worker exposure study or re-entry study calls for only three work-exposure days during the study, a set of field fortification samples should be performed on each of the work days to provide enough field fortification samples to provide adequate quality assurance for the study. If there are a number of discrete workdays within a worker exposure study, a number of field fortification sets should be run to provide an adequate range of weather conditions under which the test is performed. In this instance, one field fortification set should be performed on the first day of worker exposure, others at intermediate times during the field phase of the study, and one set on the final day of exposure.

The make-up of a set of field fortification samples at each fortification event should be considered. Usually, a triplicate set of matrix samples fortified at a 'low concentration' of test active ingredient and a triplicate set of matrix samples fortified at a 'high

concentration' of test active ingredient should be adequate to define the stability of the active ingredient under field conditions. These 'low' and 'high' concentrations should bracket, as far as possible, the concentrations of the active ingredient expected on the matrices in the field. The concentrations of active ingredient expected on the matrices are usually hard to predict and some room for error in this guessing process should be allowed.

Another consideration when planning field fortification levels for the matrices is the lowest level for fortification. The low-level fortification samples should be set high enough above the limit of quantitation (LOQ) of the analyte so as to ensure that inadvertent field contamination does not add to and does not drive up the field recovery of the low-fortification samples. Setting the low field fortification level too low will lead to unacceptably high levels of the analyte in low field spike matrix samples if inadvertent aerial drift or pesticide transport occurs in and around where the field fortification samples are located. Such inadvertent aerial drift or transport is extremely hard to avoid since wind shifts and temperature inversions commonly occur during mixer–loader/re-entry exposure studies.

(d) Placement of field fortification matrices at the field site

The placement of the fortified matrices is important. The location or placement of the field fortification samples relative to the treated test area has been the subject of some disagreement among scientists who perform such studies. One should keep in mind that the purpose of field fortification samples is to measure the field stability of the active ingredient on certain matrices used in the exposure study. Hence the fortified samples should be kept far enough away from the area treated with the pesticide to ensure that aerial drift or aerial transport of the pesticide does not contaminate the field fortification samples. As discussed above, any amount of contamination can add unwanted active ingredient to the matrix and drive up the recovery of the active ingredient from the matrix. This is undesirable in worker exposure and re-entry studies. A compromise must be made between placing the field fortification samples too close to the treated test area and placing them unrealistically so far away as to avoid localized climatic factors at the test site.

Field fortification samples such as patches, inner/outer dosimeter sections, air tubes or filters and other matrices are usually placed on a covered table near the area treated with the pesticide. Prior to placement of the dosimeter sections on the table, the table should be covered with new unused sheets of aluminum foil, plastic sheeting or disposable paper, which serves to ensure that the dosimeter sections are free from any previous contamination that may have been on the table. The table may also have a layer of sponge board or cork under the aluminum foil to allow for pinning the dosimeter sections to the table to avoid the wind blowing the dosimeter sections from the table. The dosimeter matrices or patches can then be pinned to the tabletop to avoid wind blowing them away. The dosimeter pieces or patches are organized in the field so that the matrices to be fortified at the high rate are located on a separate table about 20–30 ft and downwind from the matrices to be fortified at the low level of active ingredient.

Field-fortified air tubes or filters attached to air sampling pumps should not be placed on tables having other spiked matrices since volatilization of the active ingredient from the other matrices may lead to contamination of the spiked air samples.

Care should be taken to have plastic sheeting available to cover the tables if sudden rain occurs. Alternatively, the tables with the matrices may be moved under the cover of a shed in the case of rain. Some would argue that the matrices should be left in the rain to mimic the exact conditions of the test site; however, generally, the workers wearing the test dosimeters will not work in the rain or the researcher may decide to bring the workers out of the rain in order to have more control of the interpretation of the results of the study at a later time. Generally, more experienced researchers will opt for removing the field fortifications and workers from a rainy situation.

Control field matrices are usually placed at the field site upwind and at a significant distance from the spray or re-entry area so as to avoid all obvious routes of contamination at the test site that may destroy the integrity of the control samples. However, the control matrices should not be placed so far away from the test site as to avoid any suspected contamination that might occur from drift or other sources of contamination. One may want to define better the conditions at the test site in order to interpret better the exposure data collected from the volunteers' matrices.

(e) Fortification of dosimeter matrices

Matrices such as inner/outer dosimeter sections should be fortified as follows. The dosimeter section should be folded to create at least three layers of cloth and laid on a piece of aluminum foil large enough to use as a wrapper for the dosimeter section as the sample is being collected at a later time. This foil will allow for the capture and collection of the entire amount of the active ingredient if the fortification solution inadvertently penetrates all the cloth layers and deposits some of the fortification solution on to the aluminum foil surface. The fortification solution should be delivered on to the cloth surface using a volumetric pipet. Usually, a 1-mL volumetric pipet is used since this allows for a reasonably low volume of the fortification solution to be delivered on to the cloth. Too large a volume will cause the fortification solution to penetrate through the cloth layers in an unacceptable fashion. Too small a volume of fortification solution will increase the error in delivering the appropriate amount of active ingredient to the cloth surface. As the fortification solution is delivered to the surface, the pipet should be moved in circles over the top layer of cloth to disperse the fortification solution evenly over the surface of the matrix.

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Inner dosimeter sections should be covered with outer dosimeter material to simulate the environment of the inner dosimeter on the volunteer, which would normally be covered by the outer dosimeter. This procedure can be accomplished by pinning the outer dosimeter material over the inner dosimeter section using straight pins. Outer dosimeter sections, patches, or other exposed matrices should not be covered.

Patches should be fortified in a similar fashion to the dosimeter sections. Care should be taken when designing a patch to make sure the patch has backing made of aluminum foil or other nonpenetrable material so as not to lose fortification solution during the fortification process. Patches representing inner exposure can also be covered with a cotton/chambray material after fortification.

(f) Fortification of air sorbent tubes and air filters

Air sorbent tubes, air filters, and OHSA (Occupational Safety and Health Administration) vertical sampler (OVS) air tubes should be fortified using a microsyringe with the appropriate amount of active ingredient. A 25-μL syringe is optimum for

delivering 20–25 μL of the fortification solution on to the air tube or air filter. Too large a volume of the fortification solution is undesirable since such a large volume will be hard to dry on the resin or filter and may cause the air tube or filter to experience breakthrough of the analyte into the back part of the air tube resin. In addition, this may over-saturate the air filter beyond its capacity, causing breakdown or disruption of the air filter's integrity. The fortification solution should be carefully delivered on to the air tube resin to avoid penetration of more than a few millimeters beyond the surface of the resin. When fortifying an air filter, deliver the fortification solution slowly from the syringe and move the syringe in a circular motion over the filter in order to distribute the fortification solution over a large portion of the filter surface. Do not penetrate or scratch the surface of the filter.

After fortification, the air tube or air filter is then left upright to dry for about 15–30 min prior to turning on the attached air monitoring pumps. Air monitoring pumps with fortified air tubes or filters should be placed in a downward position on a table away from the treated field and the pump run for about as long as the test volunteers are working in the field.

(g) Field fortification of hand wash and face wipe matrices

Hand wash matrices should be fortified in the field as follows.

Jars containing the same amount of hand wash solution as used to collect the entire hand wash from the test volunteer should be fortified. The samples are fortified with the appropriate amount of active ingredient solution using a 1-mL volumetric pipet, blowing out the remaining solution in the pipet. The solutions are capped, shaken, and placed immediately in a freezer or dry-ice cooler.

Face wipe samples are treated similarly. The face wipe is placed in an appropriate jar and wet with the appropriate amount of wash solution. The sample is then spiked using a 1-mL volumetric pipet and immediately capped, processed, and frozen.

A short weathering time for hand wash and face wipe samples is appropriate since these types of samples taken from test volunteers are usually processed and frozen immediately and are not subjected to weathering as are the dosimeter or air matrices.

(h) Field fortification of biological fluid samples

Field fortification of urine and other types of biological fluids should be carried out as follows.

Control urine should be collected from individuals who have no apparent past history of exposure to the active ingredient. This control urine must be stored frozen until used for field fortification purposes. The urine is then thawed, shaken well, and a certain amount should be aliquoted into a small jar/bottle to use for field fortification. The active ingredient is then added to the urine using a 1-mL volumetric pipet, the solution is shaken well, and the sample is immediately frozen. Occasionally, the fortified sample can be left at room temperature or at some lower temperature in a liquid state to simulate field storage during collection of the urine sample. After leaving the sample at such temperatures for the prescribed length of time, the sample is immediately stored frozen.

(i) Weathering field fortifications

Study matrices fortified and left under field conditions are said to be 'weathered'. The study matrices are usually weathered for a length of time equivalent or near to the

time during which the test volunteers are exposed to the test pesticide during the day of monitoring.

The time at which all matrix samples are fortified, the time that the air pumps are turned on and off, and the time that all matrix samples are processed after weathering should be recorded in the raw data.

(j) Choosing, preparing, maintaining and shipping field fortification solutions

The preparation, maintenance, and integrity of field fortification solutions during a worker exposure/re-entry field study are of the utmost importance in maintaining the integrity of such a study.

One of the most important considerations when planning the field fortification portion of a worker exposure/re-entry field study is the selection of the test product active ingredient. Most test products contain active ingredients which are suitable for field fortification either as an aqueous suspension or as the technical grade active ingredient dissolved in water or an organic solvent. However, there are some active ingredients which are not stable on weathered matrices, and this leads to low recoveries under field conditions. The use of these unstable active ingredients in a worker exposure/re-entry study can lead to some serious problems in interpretation of the field recoveries from field-fortified samples. For this reason, direct dermal exposure studies using dosimeters with such unstable active ingredients are not recommended. In such cases, one should consider other methods to determine exposure such as biological monitoring. One way to determine whether the test substance active ingredient is going to be suitable for field fortification of dosimeter or air tube matrices is to perform a field pilot study where the active ingredient is applied at various concentrations to the matrices to be used in the proposed field study, and the matrices are allowed to weather for several hours outdoors in extreme conditions of sunlight and heat. Both the active ingredient in water or organic solvent as well as the test product suspended in water (or other carrier) should be tested on matrices in the field. Such pilot studies will answer the stability questions of the test product in question and allow one to make more rational decisions about which method of exposure to use in the field.

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One issue that is of some importance when considering the makeup of field fortification solutions is whether to use the technical form of the active ingredient in solvent or the formulated test product in a carrier such as water. This issue has been a point of contention for many years among scientists who perform and evaluate such studies. There are some advantages and disadvantages to either choice.

The use of formulated material (generally suspended in water) allows the researcher to work with the form of the test material that will be the most commonly encountered under field conditions. The formulated material would be found under most circumstances on field surfaces and in the air after treatment of the field with the test product. The greatest problem with the use of formulated product in water as a field fortification suspension is the maintenance of the homogeneity of the field fortification suspension. To maintain the homogeneity of the active ingredient in the field fortification suspension, one should shake the field fortification suspension vigorously for at least one minute and immediately withdraw the aliquot for the field spike from the fortification suspension just prior to fortification of the sample.

Another disadvantage of using the formulated product in a carrier for field fortification of dosimeters or air samples is the required increased drying time of the spike on the fabric or sorbent. When the spike is applied to dosimeter material as an aqueous suspension, drying takes several minutes. This can lead to runoff and loss of the spike from the garment material. Care must be taken to be aware of this phenomenon and to take action to avoid this problem. Some things can be done to mitigate this problem, e.g., placing the fortification sample into the garment and letting it soak into the material before exposing it to the weathering conditions.

When fortifying air tubes or air filters, the use of the formulated active ingredient in water is not recommended since the material on the air tube or filter must dry before air is drawn over the matrix. If the field fortification is not dry on the sorbent or air filter, breakthrough of the fortification solution may occur through the air filter or air tube into the back portion of the air tube and invalidate the procedure.

The use of the technical form of the active ingredient as a field fortification solution has some advantages, although the greatest disadvantage is that the use of the technical material does not mimic the actual form in which the test product is likely to exist in the field after application of the test product. The advantages of using the technical material in a true solution with solvent allows homogeneity to be easily maintained in the fortification procedure and allows for drying to occur easily on matrices preventing runoff and breakthrough as described above.

The preparation of the field fortification solutions is of equal importance in planning the field portion of a worker exposure/re-entry study. When preparing field fortification solutions the following techniques are recommended. Solutions of technical test material in organic solvent or the formulated test material suspended in water should be made up in the laboratory using primary stock solutions/suspensions and making serial dilutions to the appropriate concentrations to be used in the field. Three replicate solutions/suspensions of each concentration of the test material should be made up and labeled A, B, and C. Replicate solution/suspension A should be used to prepare the fortification solution in the field while replicate B should be a 'travel fortification solution/suspension' to be handled in the field precisely as the A replicate is handled except that no material is to be withdrawn from the solution/suspension. This replicate B solution/suspension can be refrigerated/frozen, taken out, opened and exposed to the same environmental conditions as replicate A solution/suspension was during the course of the field research. The replicate A may then be shipped back to the analytical laboratory to determine the relative stability of the field fortification solution during the course of the study. Replicate field fortification solution/suspension C should be kept refrigerated/frozen at the field site as a backup to solution/suspension A.

One alternative method for preparing field fortifications solutions/suspensions is to prepare each fortification sample of each matrix in a separate mini-vial in the analytical laboratory and ship the vials to the field for use. This procedure precludes the use of pipets in the field and may be useful when Field Scientists not experienced in the use of pipets are involved in the field fortification process. One disadvantage of this procedure is that the mini-vials, if not designed correctly, will be hard to handle in the field, and surface tension of the suspension or fortification solution will tend to leave unacceptable amounts of the solution/suspension in the vial or at the lip of the vial and not on the matrix in question. This procedure may lead to cross-contamination of samples as the field fortification liquid is forced from the top

of the vial during fortification and inadvertently deposits on the glove finger of the Field Scientist performing the fortification process. Alternative means to remove the liquid fortification solution from the mini-vial should be considered, such as use of a disposable Pasteur pipet to remove the liquid from each vial and deposit the liquid on the surface of the matrix. The remaining contents of the vial can be washed on to the matrix using a small amount of water delivered from a clean Pasteur pipet. A new Pasteur pipet should be used for each vial to avoid cross-contamination of the wash water.

Shipment and maintenance of the integrity of the field fortifications solutions are extremely important for worker exposure/re-entry field studies. Field fortification solutions/suspensions should be shipped refrigerated on blue ice or frozen on dry-ice to the field. Ensuring the delivery of the field fortification solutions/suspensions to the right location and at the right time is important. Care must be taken in this endeavor since overnight delivery services can impact the study tremendously by not delivering the field fortifications to the right place on time. Avoid shipping on weekends since the package may end up being delayed and subjected to extreme temperatures for long periods, compromising the integrity of the solutions/suspensions. Chain of custody forms must accompany all shipments. These should arrive with the samples and be used to document the condition of the solutions/suspensions upon arrival at the field site.

Maintenance of the integrity of the field fortification solutions while at the field site is another important consideration during the course of any field worker exposure/re-entry study. Field fortification solutions/suspensions should be kept under laboratory recommended conditions in a freezer or refrigerator or at ambient conditions as required to maintain the maximum stability of the test active ingredient. Maximum and minimum daily temperatures should be monitored in the storage units of the field fortification solutions and emergency measures should be in place in case of power failures or other circumstances which may lead to the compromise of the stability of the test active ingredient. For example, a portable generator should be kept in the field to provide for emergency power if the freezer/refrigerator fails. A nearby source of dry-ice should be sought and be available in case an emergency generator is not available.

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(k) Preparing field fortification samples in the field vs laboratory

Some more recent field techniques have focused on the location of the preparation of field fortification samples and have taken some of the responsibility for the preparation of the field fortification samples from the field personnel and placed them with the analytical laboratory. For example, it is becoming more common for the analytical laboratory to prepare air sample field fortifications in the analytical laboratory, freeze them, and ship them to the field for use in a frozen state. Whereas there may be some advantage to this technique in that the air tube fortification samples may possibly be fortified more accurately in the laboratory under controlled conditions than if done in the field, there are some inherent scientific problems with this method. First, one reason for the field fortification is to test the ruggedness of the field techniques of the researcher under extreme field conditions. Second, the act of freezing and thawing the sorbent matrix within the air tube itself may have an impact on the recovery of the analyte from the air tube after exposing the sorbent to field conditions

and extreme air flows produced by the air sampling pumps. While such techniques may have some short-term administrative value to cut costs or cut down on field personnel, the scientific validity of such techniques must be demonstrated under controlled laboratory conditions for each matrix and each analyte prior to taking this method to the field level.

(l) Travel fortification samples

Travel fortification samples are a type of field fortification that is usually prepared in the field to allow the investigator to determine the stability of the active ingredient on matrices without weathering. Such matrices are fortified and placed immediately in frozen storage. Usually, one set of travel fortification samples for each matrix is prepared for each five sets of weathered field fortification samples. The samples are then stored and shipped using the same procedures as all other samples prepared in the field.

(m) Storage and shipment of field fortification samples

Field fortification samples are stored under various conditions in the field. Generally, after the weathering period is complete, the field fortification samples such as dosimeter sections are wrapped in aluminum foil, placed in a pre-labeled zip-type bag, and immediately placed on dry-ice in a cooler or in a freezer. Field fortification samples such as hand washes or face wipes are prepared in labeled jars, the lids are immediately taped with electrical tape, and the jars are placed in a zip-type bag and wrapped in bubble-pack and immediately placed in frozen storage. Air tubes or air filters are collected after weathering and wrapped so as to prevent breakage. These samples are then placed in a pre-labeled zip-type bag and immediately placed in frozen storage.

Field fortification samples may be shipped with field samples but not with controls. Controls should be kept separate from treated samples and may be placed in a separate container within the container used to ship the treated samples. Samples shipped overnight should be shipped in coolers with sufficient dry-ice to maintain the samples in a frozen state for at least 48 h in case a delay in shipment of the samples occurs. Samples should not all be shipped together in one shipment but should be split to ensure that all the samples would not be lost at the same time. A chain of custody form should accompany each separate cooler or shipping box and should list each sample that is in each box. The receiver of the shipment should fill out the chain of custody form and record the conditions of the samples upon arrival at the analytical laboratory indicating whether or not the samples were frozen, ambient, or otherwise upon arrival and if the sample integrity had been compromised during shipping.

2.5.4 Techniques for measuring worker exposure and re-entry exposure: indirect measurement by biological monitoring techniques (urine)

The use of biological monitoring for the determination of exposure levels of pesticides to farm workers has historically been a very important method. Studies of exposure of workers to various pesticides utilizing biological monitoring have been published widely in the literature.[21–24] Biological monitoring measures the amount of the pesticide or metabolite in the urine or blood, and such measurements are useful in estimating indirectly how much exposure has occurred to an individual over a period of time.

(1) Prerequisite studies for a successful biological monitoring study

In order to be able to interpret biological monitoring worker exposure study results, there must be good scientific background information on the metabolism, pharmacokinetics, and excretion pattern of the active ingredient in test animals and/or in humans. A thorough discussion of the details of procedures to be used to understand the complex nature of metabolism and excretion patterns for the active ingredient is beyond the scope of this article. However, there is a wealth of information concerning such methods in the literature. The importance of having this background information on hand cannot be understated since the pharmacokinetics and excretion pattern will allow the researcher to set the interval at which urine, blood, or other biological samples may be taken to gather definitive information on the body burden of the active ingredient. In addition, the metabolism will define which analytes to monitor in the biological sample. For example, pesticides excreted as the parent or just a few high-percentage metabolites in the urine are good candidates for determining exposure using biological monitoring techniques. Pesticides that are excreted as many low-percentage metabolites are not good candidates for using biological monitoring unless one can employ a common moiety approach for determining the mix of metabolites in the urine.

(2) The duration of sampling and size of biological samples used to monitor pesticide exposure in farm workers

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The duration of collection of biological samples from farm workers is determined by the excretion pattern of the active ingredient or its metabolites. Generally, collection will encompass a period of time prior to exposure to about 1–3 days beyond exposure. Background samples should be taken from workers for the 24 h prior to the first application of the test product. This will allow an up-to-date examination of the background levels of the parent or metabolites in the worker's urine.

The selection process of volunteers for the study should take into account any aberrant background levels of the parent or metabolites in their urine. For this reason, the researcher may want to take a background urine sample from the worker about 2 weeks prior to the application of the test product and have the sample analyzed for the parent or metabolites in question. This will allow the researcher to determine if the worker is 'clean' enough to be used in the study since one would not want to have a volunteer in the study with unacceptable detectable levels of the parent or metabolite in the urine. If the workers are found to be 'clean' at this point, the workers must not come in contact with the active ingredient prior to the application day of the test substance. In practical terms, this approach is not used all the time since workers who are actively applying pesticides for commercial operations or even on an individual basis on a farm do not wait for the researcher to examine their urine. The need to apply pesticides to crops at certain times of the year (e.g., at planting or at a certain growth stage) or under certain weather conditions (e.g., before rain sets in or waiting for days for fields to dry) usually supersedes any attempt to regiment the worker test subjects to obtain urine or blood samples.

(3) Collection of urine samples from test subjects

The sample size for any biological sample depends on two criteria: (1) the amount of sample needed by the analytical laboratory and (2) the portion of the excretion pattern that the researcher is interested in observing.

When considering how much urine to collect, one must decide whether to collect individual voids as discrete samples or to collect larger samples where the test subject voids several times in one collection vessel. If the researcher is interested in examining the analyte in the urine in each void, smaller 500-mL wide-mouthed jars can be used to collect each void over a 24-h period. These are generally referred to as 'spot' void samples. Taking void spot samples in this manner allows the researcher to examine each void for the test analyte and also to composite a portion of each void into one 24-h sample in order to look at the overall concentration of the test analyte in the 24-h urine sample. If the spot samples are to be composited, aliquots of each spot sample should be removed based on each void's percentage of the total weight of the 24-h sample. The spot sample aliquots can be composited to form one 24-h sample. The leftover spot samples can be used to obtain individual void measurements of the analyte in question.

Another option for the researcher is to collect two 12-h urine samples each in 4-L polyethylene urine collection vessels or in large 1-L wide-mouthed polyethylene jars. This allows the researcher to examine the excretion pattern of the active ingredient in two 12-h segments.

When collecting urine samples from test subjects, the tare weight of the collection vessel must be recorded before giving the container to the volunteer. The urine samples must be kept cold during the collection process. To do this, the samples should be kept in Coleman-type coolers with blue ice in the bottom. The blue ice should be frozen solid prior to placement in the cooler. Usually 3–5 pieces of blue ice should be used per cooler.

Dry-ice has been used on occasion in attempts to freeze the urine as the sample is collected. About 1 kg of dry-ice is needed in each cooler to make sure the dry-ice does not sublime and disappear during the collection process. When using dry-ice, a combination of blue ice and dry-ice will ensure that the samples remain at least at cool temperatures throughout the 24-h sampling period if the dry-ice disappears.

Marking collection jars or vessels and coolers is an important aspect of the collection process. Sample jars or vessels must have a label which has a space for the volunteer's name, date of collection of the sample, and the time that the sample was collected. The volunteers are usually instructed to write this information on the label when the sample is provided.

Coolers are usually marked for each test subject with a piece of tape on the top or side of the cooler which indicates the study number, cooler number, and test subject's name. The time that the coolers are delivered to and retrieved from the test subject is recorded by the Field Scientist.

(4) Volunteer compliance when taking urine and blood samples

One of the most difficult and frustrating portions of a biological monitoring study is the compliance of the test subject when collecting a 24-h urine sample. Some volunteers will comply fully with the Field Scientist and provide all voids within the

24-h time frame. This degree of compliance is not the norm. Usually, there are many factors which prohibit a test subject from providing a true 24-h sample. The main factor is the inability of the volunteer to take the study seriously. The researcher must take great care to convince the test subject of the importance of the study. The second factor is the inability of the worker to carry his/her cooler to work, school, or other situations such as ball games. In many instances the worker is embarrassed to take the coolers to such places and consequently will miss one or more voids over the course of 24 h. Many solutions have been attempted to overcome this problem, e.g., giving more compensation to the worker or sequestering the volunteers in a motel for the length of the study period in order to have more control over the collection process.

Since collection of all urine from volunteers over a 24-h period may not be possible, creatinine analysis of the composite total urine sample is recommended. This will allow for a more scientific analysis and interpretation of the excretion pattern presented by the worker during the course of the monitoring.

Taking blood samples from volunteers is relatively easy, once they have given their consent, compared with taking urine samples. Usually, the volunteers are transported by a member of the field scientific team to a clinic near the test site where the blood samples are withdrawn and preserved by a trained certified professional nurse or doctor. The samples can then be preserved and sent to the clinical laboratory or analytical laboratory by the clinic or by the field research team.

(5) Processing, storage and shipment of urine samples

After collection of the urine samples from the test volunteers, the samples should be processed as follows. The urine samples are brought back to a field laboratory where they are weighed. The weight, date, and time of each void are recorded. If compositing is necessary at this point, the compositing is completed prior to freezing the samples. The labels on the samples are then secured by placing clear tape over them, and the lids of the jars are secured with electrical tape. The jars are placed in zip-type bags and subjected to freezer conditions for storage. The time placed in the freezer and the freezer location for each sample are also recorded. Maximum/minimum freezer temperatures are recorded daily to document the conditions of storage of the urine samples.

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Urine samples are shipped as described for all samples discussed above, using appropriate amounts of dry-ice to keep the samples frozen during shipping and by providing a chain of custody form listing each sample number during shipping. Control samples are to be kept separate from treated samples during shipment to the analytical laboratory.

2.5.5 Methods for measuring worker exposure using simultaneous biological monitoring and whole-body dosimetry

Simultaneous biological monitoring and whole-body dosimetry form a technique which has been developed in recent years to compare worker exposure data collected using whole-body dermal dosimeters and simultaneous collection of urine from the same worker during the same time frame of exposure. Researchers are beginning to show that the two once-considered divergent methods of measuring worker exposure are actually comparable using simultaneous complementary techniques with the same

worker.[3,25] Some of the field techniques which have been used in the past to carry out such studies are described below.

(1) Type and preparation of whole-body dosimeters

The choice of whole-body dosimeters when performing simultaneous biological monitoring and dosimetry studies is of extreme importance. The basis for the technique itself lies in the purpose and choice of the whole-body dosimeter. An outer garment (outer whole-body dosimeter) should be chosen which is the type of garment worn by the worker under normal circumstances during the work period. The garment may be an outer short- or long-sleeve shirt and pants or may be a coverall worn over a shirt and pants. If a coverall is worn over a shirt and pants, the coverall is considered to be the outer dosimeter. The inner dosimeter is usually a tee shirt and brief to be worn under the outer dosimeter by the test subject. No long underwear inner dosimeter is recommended while using this technique, since the long underwear will unnecessarily block the penetration of the active ingredient through the skin and make the interpretation of the final data difficult. Both outer and inner dosimeters may have to be pre-extracted or processed as described in a previous section to remove any analytical interference in the garments.

(2) Use of hand washes, face wipes, or head patches to measure hand, face, and neck exposure

The use of hand washes to measure hand exposure to workers during the use of simultaneous dermal dosimetry and biological monitoring is of value to the technique itself. One can assume that most agricultural workers will wash their hands at least once if not twice during the course of the day One must consider if a worker washes his/her hands during the course of the day using soap and water that a portion of the potential dermal exposure to the individual will be lost and will not be absorbed through the hands and would not be a part of the exposure to the worker as determined by biological monitoring. Collecting Aerosol OT/water hand washes from the worker just prior to the first exposure of the day, at the lunch break, and at the end of the work replicate allows the researcher to collect dermal exposure data without compromising any biological monitoring data that could be obtained by urine collection from the same individual during the exposure period.

The use of face wipes, while useful for measuring face and neck exposure directly, may not be of value when using the simultaneous method and may in fact lead to an underestimation of the internal dose when collecting urine-monitoring data since a worker would not likely wash his/her face prior to lunch or dinner. An alternative method, that of cellulose patches (4×4 in) has been used effectively to predict head and face exposure when estimating the neck and face component of the dermal side of the equation while performing simultaneous dermal dosimetry–biological monitoring (SDDBM) studies. The patches are usually placed on the front and back of a cap or protective headgear that the worker wears throughout the exposure period of the fieldwork. The patches are collected at the end of the exposure period, placed face to face as one sample and wrapped in aluminum foil before freezing. The two patches are then analyzed as one sample to provide the face/neck exposure component of the dermal exposure calculation for the study.

(3) Collection of urine samples during the course of simultaneous dosimetry–biological monitoring studies

The collection, processing, storage, and shipment of urine samples during the course of the simultaneous dermal dosimetry biological monitoring studies utilizes the same techniques as discussed in a previous section. The background samples and the collection of 24-h urine samples are of utmost importance during the performance of simultaneous whole-body dosimetry and biological monitoring studies. As described previously, urine samples are usually collected as two 12-h samples in appropriate vessels in order to obtain a good representative sample. Urine samples may also be collected over a 24-h period as discrete samples, using a different wide-mouthed plastic or glass jar to collect each void from the volunteer over a 24-h period. A composite 24-h sample can then be made from each discrete void by compositing portions of each void based on the percentage weight of each void as it relates to the summed weights of each void sample. Each of these collection techniques has its advantages and disadvantages. The use of two 12-h samples allows the researcher to examine a 'whole urine 24-h sample' and is much more cost-effective to perform, whereas the latter technique allows one to examine each void as it relates to the excretion pattern of the parent or metabolite(s) from the body.

Whichever technique is used, it is recommended that urine samples from volunteers be taken for an extended period of time that encompasses the entire post-exposure excretion of the active ingredient or its metabolites. In addition, it is recommended that the study volunteer refrain from handling the active ingredient for an extended period after the application phase or re-entry phase of the study is completed and until the collection of urine samples is completed. This will allow for the excretion pattern of the active ingredient or metabolites to achieve a profile that is more easily interpreted by the investigator.

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(4) Collection of air samples during simultaneous dermal dosimetry–biological monitoring studies

The collection of air samples using air tubes and/or filters is of value during the course of the field research when performing SDDBM studies. The air tube/air filter data can be used to estimate the portion of the total body burden, which originates from respiratory exposure to the active ingredient. Of course, if an organic vapor respirator is used by the worker as a matter of course, the respiratory exposure component should be 'backed out' of the final exposure calculation.

(5) Calculation of dermal exposure and internal dose using DDBM data

The calculation of potential total dermal exposure of mixer–loaders and re-entry workers using dosimetry data and calculation of the internal dose using biological monitoring data is complex but will be discussed briefly.

(a) Potential total dermal dose

The potential total dermal dose (PTDD) for workers is a summation of the skin exposure data, the hand wash data, and the head patch data for each individual worker.

The skin exposure is first calculated by correcting the outer whole-body dosimeter residues by the penetration factor. The penetration factor is derived from dividing the total outer torso residues into the total residues on the combined tee-shirt and brief

sample from that individual. The hand wash data from the individual test volunteer are then calculated by subtracting the hand wash data for the pre-exposure hand wash from the sum of the hand washes performed throughout the course of the replicate during the work day for that individual. The head patch data for the test subject can be corrected for the total face/neck area by multiplying the head patch data in residues per square inch by the appropriate correction factor. Finally, the PTDD can be estimated by summing the skin exposure, hand wash, and face/neck exposure values.

(b) Internal dose

The internal dose (ID) for workers can be calculated by using the analytical data from the urine collection carried out simultaneously with the dosimetry. The calculation of the ID using urine data is complex and will not be dealt with in detail. However, there are several references, that can help guide one through such calculations. Nolan *et al.*[26] have addressed this subject in great detail, as have other researchers.

Calculation of ID using biological monitoring techniques requires the knowledge of the pharmacokinetics of the parent pesticide in laboratory animals. This will allow the use of the parent or its urine metabolite(s) to calculate the total amount of the parent that had been absorbed through the skin of the test subject. The amount of the residue in the urine should be corrected for any molecular weight differences between the parent and its urine metabolite(s) and also corrected for daily urine excretion volumes based on creatinine analysis of the urine samples.

Whereas the use of laboratory animals is of great value in determining the metabolism and excretion patterns of the pesticide in question, this technique can have some drawbacks, especially if the human metabolism and excretion patterns are different from those for laboratory animals for the pesticide. If no human metabolism and excretion data are available, one must be careful with the interpretation of the urine data from the workers.

2.6 *Observations of the volunteers during the conduct of the field study*

Observations of the test subjects during the course of the field portion of the worker exposure or re-entry study are extremely important in order to interpret the data that are gathered at the field site and to interpret the final analytical data. There are two schools of thought when making observations of field workers during mixer–loader/applicator worker exposure or re-entry studies.

The first school of thought centers on tight control of the activities of the volunteer during the course of his/her workday. In this case, the worker is controlled closely. The worker's activities during a typical work period are usually planned well ahead of time and dictated by the protocol or by the label of the product. For example, the worker may be reminded constantly to wear his/her gloves during mixing loading or to drive an air blast spray rig up and down rows of an orchard so the wind does not blow in his/her face. Using this technique, the researcher may want to examine what the exposure may be when the label is followed very closely or attempt to define the future label of the product in very strict terms. In addition, the researcher may be attempting to refine the field portion of the study to maintain a degree of control that allows him/her to compare various subsets of the field research for later regulatory

decision-making (e.g., mitigation of exposure through various types of protective clothing or hygienic procedures).

The second school of thought maintains much less control over the actions of the field worker during the course of the study replicate. When following this philosophy, the researcher is usually interested in looking at more realistic and typical practices of the agricultural worker. In this case, the researcher may not decide to intercede if the worker is not experienced in safety procedures and, if not required by label, decides to spray with an air blast sprayer in a fashion that would bring the spray back into his/her face. Another example of this type of control in a study is when the researcher determines that the label of the product gives the worker the option of using a closed system and not using some of the protective equipment recommended by the label. This scenario of using a closed system and less protective equipment may be allowed by the researcher as recommended by the published EPA Worker Protection Standards.

In any event, whichever philosophy is adhered to, the protocol of the study should outline exactly what procedures are to be used and the purpose for the degree of control one might want to exert under field conditions.

Observations of field activities are performed by one or more Field Scientists. Normally, each volunteer worker is observed by an individual Field Scientist. The Field Scientist must remain with the worker at all times and closely observe such activities as loading the chemical, spraying the field, harvesting, scouting, and cleanup activities. The Field Scientist should remain at a safe distance from the worker to avoid any serious exposure to the pesticide which may occur during the course of the replicate. Protective equipment may be necessary for the Field Scientist depending on expected exposure levels and the toxicity of the product. In any event, the Field Scientist should have anticipated the risk of close observation and be aware of what protective measures are necessary.

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There are situations where close-up observations of the volunteer study participant may not be warranted. For example, during a study to determine exposure to pesticides of a group of custom applicators using biological monitoring, observing the workers may not be acceptable in order to make sure that the exposure levels are not biased by any control of the study by the investigator.

2.7 Data collection and the use of field forms

Field forms are extremely important for collection of field data during the course of a farm worker exposure study. The following is a list of important uses of field forms for farm worker exposure studies.

1. The use of field forms documents all the observations of the activities of the workers for future reference. The observations may be used to revisit exposure events and help define protective equipment measures for the pesticide label.
2. Field forms are necessary if GLP standards are to be followed. Field forms can be developed in such a manner as to document that each step of the protocol was followed during the course of the field execution of the study.
3. Field forms should be used to document the movement of the test chemical from the point of manufacture to the field, documenting the name brand, chemical name, Chemical Abstracts Service (CAS) number of the active ingredient, composition,

and other important information which may be necessary to know at a later date when reviewing the study.

4. Field forms can be used to document the fortification of the matrices during the field-fortifying phase of the study. The matrix fortified, the sample number, the identification and the amount of fortification solution used, the time fortified, and the time that the matrices were removed from the field and stored should be documented.
5. Field forms may be used to document the movement of the field samples from the field to the analytical laboratory. Chain of custody forms may document the sample number, when it was sampled, when it was shipped, where it was being shipped from, and where it was going. The chain of custody form may also contain information on when the sample was received, by whom it was received, and the condition of the samples upon arrival at the laboratory. The chain of custody form also documents that the sample itself was taken.
6. Field forms may also be used to document the movement of samples in and out of storage, whether in a freezer or held at ambient temperature. Field forms have also been used to document the environmental conditions for storage of the samples.

There are many other uses of field forms which are not discussed here but which are critical to the success of a farm worker exposure study.

2.8 Storage and shipping of study samples

Samples of dosimeters, hand wash, face wipes, patches, air tubes, filters, etc., should be immediately frozen in the field by placing them in coolers of dry-ice or in freezers immediately after collection. If dry-ice is to be used, enough dry-ice should be present in the cooler to freeze the sample within 15–30 min.

Freezers should be used to maintain long-term storage of the matrices. Freezer temperatures should be monitored using max/min thermometers or recording thermometers that will be durable at $-10\,^{\circ}C$. Daily recordings of max/min temperatures are necessary to ensure that the freezers are maintaining sufficient temperatures to keep the samples frozen. Back-up generators or dry-ice should be available in case the power to the freezer fails during storage of the samples.

Samples should be shipped under chain of custody (as discussed above) using an overnight courier service or freezer truck. Advantages of using freezer trucks are obvious since overnight couriers do not document environmental conditions during shipping as with the freezer truck.

3 Making sense of field data from worker exposure and re-entry studies

3.1 Organizing field data

Field data from a worker exposure and re-entry study can be organized many ways. Field data usually fit into several categories including activities and traits of workers

(age, weight, height, etc.), air pump data, field-fortification data (amounts and times of fortifications), weather data, hand wash and face wipe sampling times, etc.

3.2 Correcting field and analytical data

There are a number of ways to display and correct field and analytical data, and a few will be discussed here. However, the omission of any technique for handling data does not imply that the techniques may be faulty.

3.2.1 Dosimeter data

Dosimeter analytical data usually do not need any correction. One may choose to subtract the background levels of the analyte found in control field dosimeters from the analytical value of the field samples themselves. This is certainly optional and if not done should be noted in the field raw data.

3.2.2 Hand wash/face wipe data

When multiple hand washes or multiple face wipes are performed in the course of a replicate, the analytical results from the multiple washes of the matrix are added. Occasionally, a hand wash or face wipe is performed on a worker prior to the replicate to determine the background hand level of the analyte (active ingredient). If this is the case, the background amount can be subtracted from the overall analytical result for that wash.

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3.2.3 Correcting for field recoveries

The analytical values for the active ingredient found in the matrices of the study can be corrected for the field-fortification recovery by dividing each matrix analytical value by the average recovery of the field fortification samples for that matrix. In some cases, the high and low recoveries for the matrix are averaged, and that average value is used to correct the field sample matrix. Occasionally, the average of the low or average of the high field fortification samples may be used to correct an analytical value of the matrix since the low or high concentration fortification recovery may reflect the analytical value of the matrix in question. Several variations of the two techniques have also been used in the past.

4 Conclusions

Many and varied field techniques involved in the planning, execution, and direction of the field portion of worker exposure and re-entry studies have been considered. Suffice it to say that there are many ways to perform such studies, and the important thing to remember is that good scientific thought and planning will produce an excellent study. The scientific validity of such studies should rest on the basic principles of science. There are various guidelines and protocols which may be followed for regulatory

purposes. However, in the final analysis, such field studies should stand solely on their scientific merit.

References

1. H.R. Wolfe, W.F. Durham, and J.F. Armstrong, Arch. Environ. Health, **14**, 622 (1967).
2. W.J. Popendorf, 'Advances in the unified field model for reentry hazards,' in "Dermal Exposure Related to Pesticide Use; Discussion of Risk Assessment," ed. R.C. Honeycutt, G. Zweig, and N.N. Ragsdale, ACS Symposium Series 273, American Chemical Society, Washington, DC, pp. 323–340 (1985).
3. R.C. Honeycutt and E.W. Day, Jr. (eds), 'Worker Exposure to Agrochemicals – Methods for Monitoring and Assessment,' Lewis Publishers, Boca Raton, FL (2000).
4. J.T. Griffiths, C.R. Stearns, and W.L. Thompson, *J. Econ. Entomol.,* **44**, 160 (1951).
5. G.S. Batchelor and K.C. Walker, *AMA Arch. Ind. Hyg.,* **10**, 522 (1954).
6. J.E. Davis, *Residue Rev.,* **75**, 33 (1980).
7. D.R. Hackathorn and D.C. Eberhart, 'Database proposal for use in predicting mixer–loader/applicator exposure,' in "Dermal Exposure Related to Pesticide Use: Discussion of Risk Assessment," ed. R.C. Honeycutt, G. Zweig, and N.N. Ragsdale, ACS Symposium Series 273, American Chemical Society, Washington, DC, pp. 341–355 (1985).
8. R.C. Honeycutt, 'The Usefulness of Farm Worker Exposure Estimates Based on Generic Data,' in "Dermal Exposure Related to Pesticide Use: Discussion of Risk Assessment," ed. R.C. Honeycutt, G. Zweig, and N.N. Ragsdale, ACS Symposium Series 273, American Chemical Society, Washington, DC, pp. 369–375 (1985).
9. J.C. Reinert and D.J. Severn, 'Dermal Exposure to Pesticides: EPA's Viewpoint,' in "Dermal Exposure Related to Pesticide Use: Discussion of Risk Assessment," ed. R.C. Honeycutt, G. Zweig, and N.N. Ragsdale, ACS Symposium Series 273, American Chemical Society, Washington, DC, pp. 357–368 (1985).
10. I.M. Abbott, C. G. Bonsall, T.B. Hart, and G.J. Turnbull, *Am. Ind. Hyg. Assoc. J.,* **48**, 167 (1987).
11. WHO, 'Field Surveys of Pesticides,' Standard Protocol VBC/82.1, World Health Organization, Geneva (1982).
12. 40 CFR, Part 160, Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA): Good Laboratory Practice Standards; Final Rule, August 17, 1989, United States Environmental Protection Agency, Washington, DC (1989).
13. Occupational and Residential Exposure Test Guidelines, OPPTS 875.1100, February 1996, 'Dermal Exposure – Outdoor,' EPA 712-C-96-262, United States Environmental Protection Agency, Washington, DC (1996).
14. Occupational and Residential Exposure Test Guidelines, OPPTS 875.1200, February 1996, 'Dermal Exposure – Indoor,' EPA 712-C-96-209, United States Environmental Protection Agency, Washington, DC (1996).
15. Occupational and Residential Exposure Test Guidelines, OPPTS 875.1300, February 1996, 'Inhalation Exposure – Outdoor,' EPA 712-C-96-263, United States Environmental Protection Agency, Washington, DC (1996).
16. Occupational and Residential Exposure Test Guidelines, OPPTS 875.1400, February 1996, 'Inhalation Exposure – Indoor,' EPA 712-C-96-213, United States Environmental Protection Agency, Washington, DC (1996).
17. Occupational and Residential Exposure Test Guidelines, OPPTS 875.1500, February 1996, 'Biological Monitoring,' EPA 712-C-96-264, United States Environmental Protection Agency, Washington, DC (1996).
18. Occupational and Residential Exposure Test Guidelines, OPPTS 875.2400, February 1996, 'Dermal Exposure,' EPA 712-C-96-269, United States Environmental Protection Agency, Washington, DC (1996).
19. Occupational and Residential Exposure Test Guidelines, OPPTS 875.2500, February 1996, 'Inhalation Exposure,' EPA 712-C-96-270, United States Environmental Protection Agency, Washington, DC (1996).

20. Occupational and Residential Exposure Test Guidelines, OPPTS 875.2600, February 1996, 'Biological Monitoring,' EPA 712-C-96-271, United States Environmental Protection Agency, Washington, DC (1996).
21. W.F. Durham and H.R. Wolfe, *Bull. WHO,* **26**, 75 (1988).
22. J.E. Davies, H.F. Enos, A. Barquet, C. Morgade, and J.X. Danauskas, 'Developments in toxicology and environmental sciences; pesticide monitoring studies. The Epidemiologic and Toxicologic Potential of Urinary Metabolites,' in "Toxicology and Occupational Medicine," ed. W.B. Deichman, Academic Press, New York, pp. 369–380 (1979).
23. K.A. Levy, S.S. Brady, and C.D. Pfaffenberger, *Bull. Environ. Contam. Toxicol.,* **27**, 235 (1981).
24. A.A.B. Swan, *Br. J. Ind. Med.,* **26**, 322 (1969).
25. G. Chester, *Ann. Occup. Hyg.,* **37**, 509 (1993).
26. R.J. Nolan, D.L. Rick, N.L. Freshour, and J.H. Saunders, *Toxicol. Appl. Pharmacol.,* **73**, 8 (1984).

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Electronic record keeping in a regulated environment

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1 Introduction

Over the past decade, capturing and reporting data in the electronic age have become a more sophisticated process. The volume of data that must be collected, calculated, used, stored, and retrieved is continuing to increase exponentially as more and more data are being generated on the global landscape. Regulatory oversight of data capturing and handling and the ability to store and retrieve these data accurately and quickly have also become more demanding. In earlier times, all data were collected, interpreted and stored on some type of paper format. Instrument printouts, as well as calculation sheets, were recorded on a nonelectronic medium. Today, most instruments and recording devices incorporate some type of electronic data capture, data reduction and formatting for viewing, and final data presentation.

Both the pharmaceutical and agrochemical industries face new challenges with the evolving regulatory requirements for electronic records and electronic submissions. The regulations are dynamic with the proposal of new rules on a regular basis, which often take the form of guidance documents as opposed to the traditional regulating process. The use of on-line systems, intranets, and networks allows for the electronic distribution of records and documents. Computer systems that create data and generate reports can now be found in nontraditional areas, such as field notebooks and automated weather stations. Topics in this article span the spectrum from electronic data capture systems in the field and in the laboratory to electronic reporting and archiving. As always, the ultimate goal in creating electronic records, in storing or archiving these data, and in submitting data electronically remains the same: the assurance of the integrity and quality of the data.

The use of electronic systems and automated electronic capture systems is ever increasing. These systems provide greater efficiency in the generation and management of records and documents than paper and manual processes. Thus, paper and manual processes are being replaced by the use of these systems. In order to prove

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that these electronic systems generate and store data appropriately, the computer and its associated software, which comprise the 'system', must be validated. Industries that operate in the regulatory environment must document their computer validation.

This article defines the criteria and processes for computer validation. Computer validation applies to all systems, including electronic capture systems in both the laboratory (scientific instrumentation) and field settings. Any system producing electronic records and documents, which regulators in the evaluation of product registration applications will use, needs to be validated.

The validation process is subject to the following: design specifications, user and performance requirements, preparation of a master plan/validation protocol (installation qualification, operational qualification, and performance qualification), execution of the protocol, preparation of a summary report, and on-going validation (and revalidation if changes are made).

The software life cycle activities extend until retirement of the software. However, in a manner of speaking, life cycle activities extend even beyond retirement since the data must be able to be reconstructed at any time during the life of the product, i.e., the archived record must always be accessible and readable even if the software is no longer commercially available or typically employed in the laboratory. Additional software validation includes implementation of the code and integration and performance testing. There also must be system security, change control procedures, audit trails, calibration, preventative maintenance, and quality assurance.

The validation umbrella not only covers in-house systems, but also covers vendor systems. Much of industry today is dependent to some extent on vendor-supplied electronic systems and, consequently, the vendor's validation of those systems. Because of this, there are validation issues to assess at the vendor including how the vendor addresses change control, testing and documentation, source code, integration, and implementation of the system during development. Implementation support also must be assessed.

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2 Management and integration of electronic records and documents

Today, much more than just data are produced electronically. Many documents needed for studies that fall under the Good Laboratory Practice (GLP) standards regulations are being managed electronically. These records include not only data, such as chromatographic data from automated electronic capture systems and raw data collected in electronic field notebooks, but also other documents, such as methods, protocols, reports and standard operating procedures (SOPs). Frequently, these records are generated, distributed, reviewed, and archived electronically.

GLP compliance for electronic records does not differ from GLP compliance for paper records. The increased access and distribution of records and documents enabled by electronic systems provide compliance challenges. There are many new questions to answer; for example, what is the difference between electronic approvals and electronic signatures? The solutions to these challenges lie in ensuring that system validation and management processes are in place, such as SOPs or procedures

outlining system administration, access, security, change control, training, and disaster planning.

The archiving of electronic data poses more specific challenges. GLP requires records retention and retrieval of archival records. Since software used for the creation of electronic records is upgraded at a rapid pace, the requirement of retrievable archived records can be difficult to comply with. Even when stored on electronic media, the records have to comply with the records retention period and be retrievable. The collection, storage, and retrieval of electronic records should address all GLP aspects, including environmental conditions to ensure the integrity of the media.

The integration of different forms of study information from various locations and sources is possible with electronic information. Both study management and quality assurance are addressing critical issues associated with this process. Study Directors must now keep track of more data and study reports than ever before. Quality assurance (QA) departments must have systems in place to audit electronic data.

2.1 Electronic reporting requirements

The regulatory world is an exhaustive one, indeed, with new regulations and guidance documents proposed on a regular basis addressing electronic information. There seems to be a lack of comprehensive regulations or guidance documents to establish a standard for reporting. A final rule on Electronic Records and Electronic Signatures was issued in March 1997[1] by the US Food and Drug Administration (FDA). This rule established regulations for acceptance of records in electronic format and for the equivalent of handwritten signatures in electronic form. The rule has, however, raised further questions. There is a need for clarity in certain areas with its implementation. Consequently, regulators are continually working with one another and with industry to establish a harmonized (international) and comprehensive set of standards. The FDA also published (for comment purposes only) a draft guidance document for industry on electronic records and signature validation in August 2001.[2]

The benefits to electronic reporting are legion. Electronic reporting can reduce expenses for both industry and governmental agencies by replacing paper processes, expediting study reviews, and reducing package preparation and study review expenses. There are, however, still many issues to address, including data integrity, confidentiality of business information, standard platforms, and procedures that allow industry to keep pace with current technologies.

Sometimes, even in this 'electronic age', paper is still the medium of choice. For retained records that must be made available to personnel responding to an emergency, paper is a better medium than electronic media, because paper can remain accessible during emergency events (e.g., power outages, fires, floods, etc.) that could render electronic records inaccessible.

2.2 Electronic data management of protocols and SOPs

The use of electronic systems to generate, retain, distribute, review, and archive standard GLP documentation, such as SOPs, protocols and protocol changes, is increasingly common. There are many benefits, including elimination of paper, ease

of distribution, increased availability, and ease of management. However, failure to consider and/or understand such basic GLP requirements as document availability and accessibility or where and when electronic signatures are required can result in electronic systems that are far less compliant and manageable than simple, old-fashioned paper.

Some fairly common mistakes made in managing electronic SOPs, protocols, and changes to them are listed below. If these can be avoided, the resulting computer system should meet the requirements of the US, Organization for Economic Co-operation and Development (OECD), and Ministry of Health, Labor, and Welfare (MHLW) GLP standards as well as the FDA's Rule on Electronic Record Keeping: Electronic Signatures.[1]

One common mistake is failing to understand when electronic signatures are required on electronic records. FDA GLPs require signatures only for the following: protocols, protocol changes, reports, QA reports, QA statements, GLP compliance statements, and manually recorded raw data. Note that neither electronic SOPs nor the electronic capture of raw data require electronic signatures. In the case of electronic data, the requirement is that the individual responsible be 'identified at the time of data input'. In the case of SOPs, the requirement is that they be 'authorized' by management. While all regulated electronic systems must comply with the pertinent electronic record keeping requirements, compliance with the electronic signature requirements is not necessary unless signatures are also required by the predicate rule. The FDA has made a clear distinction between the terms 'sign/initial' and the terms 'approve, authorize, identify'. In the first case, an electronic signature is required in electronic record keeping systems. In the second case, only the identity of the responsible individual must be clear through the use of unique user codes and other means. What this means is that while electronic protocol systems require an electronic signature, electronic SOPs systems do not. According to the FDA's Electronic Records/Electronic Signature Final Rule, electronic SOP systems must meet the following requirements:

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1. the system must be validated
2. the system must provide accurate and complete copies in human-readable form
3. information must be readily retrievable
4. access to the system must be limited and controlled
5. the system must have electronic audit trails
6. operational, authority, and device checks must be part of the system
7. system changes occur by change control procedure
8. open systems (access not controlled by system owner) require additional security measures, such as encryption.

Because signatures are required, electronic protocol systems must meet the following additional requirements:

1. the signature manifestation must include the printed, full legal name of the signer along with date, time, and meaning of the signature
2. the signature must be unique to one individual
3. the ability to apply the signature must be controlled either by one biometric or two other distinct identification components

4. the system must be secure
5. device checks must be present
6. the company using the signatures must certify to the government agency that the electronic signature is the legal equivalent of a written signature.

Some companies fail to consider that the timing of electronic authorization or electronic signature must meet GLP requirements, especially when multiple authorizations or signatures are required. In a merged protocol system where both the Study Director and the Sponsor sign the paper protocol and 'authorize' the electronic protocol, the protocol must not be noted as approved on the network without Study Director signature and authorization, even though the Sponsor has signed/authorized. GLPs require Study Director and Sponsor signature/authorization for approval. Additionally, since a merged system is being used, signing (paper) and authorization (electronic) must occur on the same date. In a fully electronic SOP system requiring only management's 'authorization' for new or revised SOPs, the SOPs must not be available on the network before authorization. An electronic SOP must not 'go live' on the network until management approval is obtained. In a fully electronic reporting system requiring electronic signature by both the Study Director and scientists, the report must not appear on the network as finalized even though the scientists have signed. Again, GLPs dictate that a report is final only upon application of the Study Director's signature.

Another common problem is the failure to understand that information supporting and tied to electronic SOPs, such as published literature, diagrams or equipment manuals, must also be available and current. For SOPs, supporting documents, such as diagrams or user manuals, should either be incorporated into the SOP or should be 'readily available' and current. These supporting documents must be clearly referenced and their use defined in the SOP. The same is true for electronic SOPs with the understanding that many current electronic systems are not yet capable of providing such supporting documentation in electronic format. In these cases, the fundamental GLP requirement somehow to tie and reference supporting documentation to the appropriate SOP, and also have the material be current and available, must not be overlooked. Such functionality should be included in the user requirements for new or enhanced systems. An alternative is to scan the supporting documents into portable document format (PDF) and attach the PDF file to the electronic document. PDF files may even be dropped into word processing documents in some cases. This would be one way to ensure that the supporting documentation is attached to the SOP and is readily accessible to each SOP user.

This brings to mind another common problem. SOPs must be readily available in areas where GLP work is conducted. Protocols also must be accessible. Even when these documents exist only in electronic form, they must still be available, especially in the laboratory and study rooms. This means that computer systems that contain these documents must be available in the laboratory and study rooms and that all personnel who conduct work according to these SOPs must have access. The same is true for protocols and protocol changes. In cases where a merged system exists, i.e., both paper and electronic media are simultaneously in use, it is perfectly acceptable that only the electronic form is available in the laboratory areas while electronic or paper documents are available in offices. The only caveat is that a process must be in place to ensure the equivalency of the electronic and paper media.

Another aspect of the GLP requirements that is often overlooked when only electronic systems are used is that, in the event of a system failure, a 'back-up' paper version should be available and reasonably located nearby. For example, should an electronic SOP system fail, it is unlikely that a government inspector will consider one paper copy of the SOP adequate for a large facility that includes field sites, animal rooms, an analytical laboratory, an immunology laboratory, and a clinical pathology laboratory.

Also, electronic SOPs and protocols must be available to staff at all test sites for multisite studies. If the electronic documents are to be available at several sites, the validation phase of the system must include functionality testing at each site. Documentation of system validation needs to be available at each test site as well. Electronic SOPs must have a limited life span when printed to avoid the use of an outdated document. This may be achieved by stamping each SOP hard copy 'Printout not valid after date xx/xx/xx'. This practice helps to ensure that system users will not retain printed SOPs long after the electronic SOP is revised. For company SOPs that are to be followed by an outside contractor who has no access to the electronic system, an alternative stamp may be used on the hard-copy SOPs that will be provided to the contractor that defines the date printed or indicates that the SOP is valid for use in a particular study. Whatever procedure is used, it must be clearly documented in an SOP.

Another common problem is the failure to understand that, when using a merged system (some combination of paper and electronic records), both are the 'real' SOPs, protocols, etc. As SOPs and protocols are transferred from paper to electronic media, frequently a signed, hard copy and an approved electronic version of the same document are maintained. Users of 'merged' systems must remember that one must be able to demonstrate that the paper and electronic versions of an SOP, protocol, etc., are exactly the same. For example, if the SOP is signed on a certain date, the electronic version must also indicate that approval occurred on the same date. This means that a procedure (i.e., SOP) for ensuring document equivalency must be available and followed.

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An important problem is that companies may fail to validate and manage electronic SOP and protocol systems to acceptable standards. Validation for such systems must include all standard components, including:

1. user requirements
2. system specifications
3. design documentation
4. validation plan
5. appropriate, well-documented testing that includes a validation protocol
6. actual testing [installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ)] and test results
7. test report
8. release of the system for use by management.

The validation package should be archived and must be readily retrievable. After validation is complete, these systems must be used and managed to GLP requirements.

This includes, at a minimum:

1. operational SOPs including procedures for use, change control and disaster recovery
2. limited access including security procedures and a list of authorized users
3. periodic testing
4. documented staff training
5. source code access information
6. system overview to include description and diagram.

Electronic SOPs must be separated from electronic guidelines or other documents when the non-SOP documents are managed to a separate standard. While government agencies expect appropriate SOPs for all GLP activities, the agencies have never advised against the use of other documents, such as guidelines or policies, as long as the difference between SOPs, guidelines, and policies is clear. Guidelines and similar documents are used in some organizations for general reference. In such cases, the guidelines may be managed more loosely than SOPs (i.e., system not fully validated or no operational SOPs exist). In these instances, the regulated, managed SOP system must be fully separated from the other documents and reside on a separate and distinct database.

Frequently, organizations develop cumbersome but compliant systems. The failure to create a user-friendly system is a common problem. When systems designers are concentrating on designing a computer system for SOPs and protocols that is fully compliant with government agency regulations, the system can easily become user-unfriendly and burdensome. When designing systems, special attention must be given to the following:

1. easy access (system and printer availability)
2. readable format/easy viewing
3. excellent sort capability
4. good table of contents
5. manageable signature/authorization process
6. trackable preparation
7. review and approval process for new or revised documents
8. sensible unique numbering systems
9. limitations on number of signatures/authorizations required (no more than two)
10. limitations on size of SOPs (not too long)
11. a well thought out, well-managed transition from paper to electronic documents.

A final problem is the failure to understand that there must be a method to archive electronically historical versions of electronic SOPs in addition to study documentation, including protocols and protocol changes. The FDA's Electronic Records/Electronic Signatures Rule clarifies this requirement. Once an electronic document is created, paper retention is simply not sufficient. Government agencies and the regulated industry fully understand that systems and processes for appropriate electronic archiving may not yet be technically adequate. Despite this, a good faith effort must be made. This effort could entail writing these electronic documents to compact disk (CD). When user requirements are developed, electronic archiving is a

function that is often overlooked. When merged systems are used, both electronic and paper versions of documents, including SOPs and protocols, must be retained since both are 'real'.

3 Management of field data and information

The electronic capture of laboratory data seems to be developing at a much more rapid pace than are the systems for the electronic capture of field trial data. The reason for this difference is that the types of field studies are much more varied than are laboratory studies. In the laboratory, electronic capture of chromatographic data can be used in a variety of regulated studies. In field applications, there are major differences among the types of data required for efficacy trials, magnitude of residue, dissipation studies, ecological effects trials, worker exposure testing, and dislodgeable foliar residue studies, just to name a few. These differences result in relatively small markets for electronic applications.

Field residue studies are conducted to set the limits of pesticide use as defined by the maximum use pattern including the maximum application rate, number of applications, interval between applications and pre-harvest interval (PHI), and to assess dietary exposure. In the USA, 1–20 trials are required per crop, depending upon the acreage grown and the importance of the crop commodity as human food. Environmental Protection Agency (EPA) Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) GLP[3] and Good Automated Laboratory Practice (GALP)[4] standards apply to these studies.

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Major registrants typically conduct as many residue trials as possible on their own research farms and contract the remainder to independent contract research companies. These contract research companies range in size from those that have several research farms to husband and wife teams with only one site. They also may include independent researchers who do not own a permanent test site but contract small plot test areas from farmers inside commercial production acreage. The goal of these trials is to conduct the study in a cropping system environment that represents commercial production systems, thereby ensuring that the raw agriculture commodities harvested represent commercially available commodities.

Performance trials are conducted to determine the use pattern required for effective pesticide performance. Much effort goes into determining the minimum effective rate. Usually, about 30 trials are required per major pest. Initial performance testing is usually conducted on company research farms. For crops and pests that cannot be handled internally, contractors are used. The differences between residue and performance trials create difficulties in designing an electronic system that can handle both study types well.

The advantages of electronic data capture and management are enticing the field research industry to adopt and develop electronic systems. Data collected electronically are much more easily summarized, tabulated, and reported than are manually collected raw data. Field reports may be checked electronically for completeness. The verification of calculations in an electronic format is much easier than manually checking each calculation in a hand-generated table. Such increases in efficiency not only save money and offer significant time savings for study sponsors, but also allow for quicker submissions for registration.

Additionally, significant savings are achieved by electronic transmission of data and documents, including study protocols and initial and final field reports, and may facilitate more timely study-status updates. The generation of electronic templates for field reports allows for automated entry of sequential trial numbers. Many of the residue study final reports may be generated easily electronically, with increasing quality from report to report. As formats and edits improve one report, these improvements are automatically included in the next study report. Database queries, either in Microsoft Excel or Access, can be developed, which almost instantaneously pull data into the report appendices and tables. When reports are available in read-only format on a local area network to which the QA unit has access, the QA review time would be much faster. All of these advances decrease the time from data generation to report finalization. Study sponsors using these database systems have noted the added cost for the software and hardware. Field contract researchers have questioned whether the advantage of using these systems in the field outweighs the economic burden to them in paying the base cost for their use. Current commercial systems do require more cost, time, and effort on the cooperators' part than traditional paper systems.

Electronic systems are also efficient for the management of field trials. Frequently, spreadsheets are used to facilitate contracting studies, ordering test substances and supplies, and generation and payment of invoices. However, there are some disadvantages to the use of electronic systems. The cost of a commercial field trial data system is significant, particularly when multiple copies are needed, which is the case with many field sites. However, the cost for a company to develop its own unique system would be even greater, and in many cases impossible, as qualified information technology (IT) personnel would be difficult to find. Even for purchased, vendor-supplied electronic record keeping systems, there are costs for system validation and testing, development and maintenance of standard operating procedures, and training. Additional costs might include the purchase of information system hardware and software, and operating system upgrades and maintenance.

The advantages of electronic record keeping for contract researchers are generally considered to be less than those for sponsor companies. Electronic checking for completeness of reports and raw data can be instantaneous for sponsor representatives. The ability to enter a given bit of information only once into a report template with instant copying to every other location where that information is needed in the report saves substantial entry and checking time. This feature of electronic systems eliminates a common source of frustration found in generating manually prepared paper reports. Electronic transmission of data from the contract researcher to the sponsor is very convenient for both contractor and sponsor. The electronic transmission (e-mail) of progress report files to the sponsor eliminates many telephone calls and is much easier than completing and faxing paper forms. E-mail is a written form of communication which can prevent miscommunication and errors and is easily retrievable. However, the use of the Internet (an open system) does present a significant data security issue unless the messages are encrypted. This is a significant GLP and electronic records compliance issue that has yet to be addressed by the industry. The use of spreadsheets to manage trial work is convenient and effective. Electronic record keeping systems can be used to generate paper reports and data copies in many cases. This is an advantage to the contractor working with sponsor companies still using paper record keeping systems. Electronic logs can be used to track reports, communications, and study activities.

The major disadvantage of electronic record keeping systems for contract researchers is the cost. A computer suitable for field use, such as a laptop with a special monitor that can be viewed in the sun, is required. For larger contract firms, multiple copies of the software and system, multiple field computers, and a network may be necessary. All personnel must be trained. As with any new technology, proficiency comes only with much practice. The frustration of learning the application and its 'quirks' replaces the frustration of entering the same data in multiple places on paper forms. The hardware of computerized systems has to be operated according to the manufacturer's specifications. The temperature, humidity, static electricity, and presence of dust in field trials often exceed the limits of the equipment's specifications. Increasingly, sponsor companies are requiring each contract facility to use a specific electronic record keeping system. Using the system may yield a substantial volume of work and capital (profit) for the contract facility. Without the system, the contractor will not be awarded the sponsor's work. Making the decision to use an electronic data collection system can be a difficult and expensive gamble, especially in today's retracting industry and greatly diminished workload to contract researchers.

When sponsor companies are using different systems, the field cooperator is then required to purchase more than one software package, thereby increasing the cost of 'doing business'. Cost increases, of course, have the greatest impact on the smallest field cooperators.

Because of the efficiency gained from the use of electronic data capture and trial management systems, one contract field company finds that it conducts almost twice as many trials as it had conducted previously and at a lower cost per trial. Not all companies find this to be true, however. Much has yet to be resolved definitively relative to the effectiveness and acceptability of electronic field notebook systems for efficacy and magnitude of residue studies. Although progress is being made on both the software and hardware fronts, user unfriendliness continues to be a complaint in the field. Several GLP compliance and system validation issues for data collection, transition, and archiving have yet to be resolved by the regulatory agencies.

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The importation of data from one electronic data system to another is improving. Some systems import weather data and sample chains of custody, such as shipping conditions, sample handling, etc., into the field raw data package. This allows for simplified reporting and tabulation. Data transmission over the Internet is improving and is already far superior to regular mail and even next-day delivery services.

4 Management of laboratory data and information

Automated electronic data capture systems have become increasingly important in the laboratory. They have improved the ease of manipulation and reporting of chromatography data. A regulatory requirement is that these systems must generate a permanent audit trail of the parameters employed in the collection and analysis of those data.

The electronic data capture systems that have become commonplace in the research and analytical laboratory allow rapid and efficient acquisition, manipulation, and reporting of vast amounts of scientific data. In addition, they have provided a means to generate a permanent audit trail which describes the conditions under which a

chromatogram was obtained, documents the identity of the analyst performing the chromatography, and records the precise time and date that the data were obtained. The data also include the time of occurrence for each event taking place during the chromatography run – for example, a gradient or temperature change.

Data collection of this type is accomplished by converting the analog output from the chromatograph to digital data readable by a desktop computer. On older systems, this was accomplished primarily by use of a data conversion box. More recently, analog-to-digital (A/D) cards are used to convert the analog output into a digital signal. The digitized data are then sent to a personal computer (PC) for processing and storage. Some newer instruments are controlled by a PC with their data output sent back to the PC directly through a serial interface card. Information stored on the hard drive of the PC can then be sent to a server where other authorized users may access this information in a variety of applications. In most organizations, data on the server are backed up on to tape or optical media on a regular schedule. The backups should be maintained at an offsite location, adding another level of security for the data.

4.1 Selection of a data system

The decision as to whether or not to purchase a laboratory automated data collection system is no longer based simply on cost. With the advent of high-throughput analyses, the increased speed and efficiency of data handling, and the advantage of having the ability to run chromatography analyses and collect and process the data on an automated system even overnight, few laboratories can compete successfully without the advantages of automated data capture.

An even more compelling advantage of automated data collection systems has developed with the advent of GLPs. These automated data systems produce an electronic record (called a meta file or meta data) of not only the chromatogram (output) generated during a chromatography run but also a record of many inputs including date, user identification, sample name and number, instrument, column type, temperatures, flow rate, etc. A dynamic audit trail can be maintained for any changes made to these meta data, including all settings, inputs, and results. When a change is made to an input parameter, a description of the change, including the time of the change and the identity of the person who made the change, is recorded electronically. The original meta data are also maintained. Additional items or comments, i.e., column pressure, also may be manually entered at any given time, with an audit trail being automatically generated for each change or addition to the original raw data. On a properly administered and GLP-compliant system, this audit trail record cannot be altered and, consequently, becomes a part of the permanent record of the entire chromatographic run.

There are many things to be considered when purchasing an automated chromatography data collection system. A needs analysis must be conducted, including a prioritization of proposed requirements and uses. No single system is the best for all situations. However, a prioritization of needs can reduce the search. Some of the major items to consider are as follows:

- can the system provide data in a usable form that will work for the tasks at hand?
- will it work with the equipment already in the laboratory, i.e., chromatographs, PCs, etc.?

- are there flexibility and expandability to meet future needs?
- can the system be used as a stand-alone unit as well as a networked unit?
- is the system user friendly and how large will the learning curve be?
- will the system meet GLP requirements?
- what kind of reputation does the manufacturer have in terms of reliability, service, etc.?
- can the company afford it?

Each of these considerations must be investigated prior to making such an important purchase. Of course, the ideal situation is that the vendor of the data capture system also produces chromatography systems, so that a complete system comprised of components specifically designed to work together may be obtained. In most cases, however, a substantial amount of equipment from various manufacturers has already been purchased and utilized in the laboratory. Budget considerations often preclude the complete replacement of working chromatography units.

The requirements of the servers in use in the laboratory must also be considered. How many separate chromatography systems can be controlled from a single server? A separate PC for each chromatography system avoids most difficulties, but in many facilities, many systems are served or controlled by a single PC server. In this case, the ability to serve and/or control multiple (up to 16) units becomes important.

4.2 System qualification

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In a GLP-compliant laboratory, a data system must meet explicit requirements guaranteeing the validity, quality, and security of the collected data. Operational qualification (OQ) must be performed after any new devices are installed in the laboratory system and whenever service or repair are performed. The role of OQ is to demonstrate that the instrument functions according to the operational specifications in its current laboratory environment. If environmental conditions are highly variable, OQ should be checked at the extremes in addition to normal ambient conditions. Performance qualification (PQ) must be performed following any new installation and whenever the configuration of the system has been changed. PQ demonstrates that the instrument performs according to the specifications appropriate for its routine use.

4.3 Access control

Any automated data collection system must include a means of controlling access to the data throughout its lifetime that encompasses its generation and storage. This access control system must guarantee that only authorized users may gain access to the specific workstations, raw data, data sources, folders, run sequences, reports, etc. The operations that are access-protected include the following: creation, modification and deletion of users, databases, directories, samples, lists, queries, server configurations, individual chromatography systems, libraries, control panels, report definitions and qualification methods. Other components also include saving and/or deletion of peak manipulations and results, starting analyses, exporting and printing data, backup and

restoration of databases, directories and sample information, import and export of data, and access to servers and individual chromatography systems.

Under adequate access control, management chooses an administrator who grants privileges and individual passwords to authorized users, thereby defining the scope of the functions that are available to each user. Whenever an individual user logs on to a system that is subject to access control using a password, his or her unique set of privileges become available to him or her.

The assignment of access and privilege can be complex. Any number of access and privilege groups can be set up with an individual being a member of one or many groups, permitting the user to have one set of privileges when dealing with one data set (e.g., a Study Director) but being restricted when dealing with a different data set (e.g., a manager).

The administrator is responsible for the handling of the function of granting accesses and privileges to users, but the actual responsibility of defining specific users' privileges and access must be decided by management and is best handled through a written SOP.

Management identifies the system administrator. This administrator may be a user or may be a member of a separate, independent department, such as IT. Each laboratory, specifically its management, must decide upon the level of security needed to protect data from unauthorized access and undocumented changes. These decisions must be clearly described in SOPs. In a GLP environment, the Study Director has ultimate control over the study and decides which users (scientists, technicians, etc.) should be granted access and privileges to study data. For example, the report writer might be granted full access to view all files and data associated with a study, but have no privileges allowing manipulation of the data.

In a GLP-compliant electronic record keeping system, original raw electronic data will not be altered, but these data can be presented and interpreted for reporting. Any changes to the raw data are documented and maintained as audit trails. The audit trail becomes a part of the raw data for the study and is archived as such.

The proper use of an automated data system, when combined with a well-managed laboratory environment, can be a substantial asset in maintaining a high level of integrity in the collection, documentation and storage of chromatographic data for government-regulated studies.

5 Metrology

Over the years, many instruments have been developed for and used in the scientific laboratory. Today, the computer is used as a major tool in the scientific laboratory for the capture, manipulation, transfer, and storage of data. Consequently, the concern for data quality has shifted from the instruments that are used in the generation of the data to these electronic systems, often neglecting the fact that the data are only as accurate as the instrument measurements. For instance, many electronic systems can be used in chromatography analysis, from the electronic log book where the test substance inventory is kept, throughout data capture in the instrument, to the digitized electronic signal that is the raw data on the computer network. For crop residue samples, the

reported residue level is only as accurate as the balance used to weigh the analytical standards on which the crop sample is quantitated.

Metrology is the science used to demonstrate that an instrument performs at a specific level of accuracy and conforms to known standards. Data generated should be reproducible and consistent. A program based on metrology principles can provide assurance that the data generated are true and accurate as measured. This means that the instrument meets performance standards and contains proper documentation of equipment qualification, calibration, and maintenance. A good metrology program with outstanding documentation practices and controls can meet the compliance needs of current Good Manufacturing Practice (cGMP)[5] or International Standardization Organization (ISO) Guide 14025 standards.[6]

To satisfy government agencies, instruments need to be adequately tested, calibrated, and/or standardized according to documented procedures. Current GLP standards state that equipment used in the generation, measurement, or assessment of data and equipment used for facility environmental control must be of appropriate design and capacity to function according to the protocol and shall be suitably located for operation, inspection, cleaning, and maintenance.

5.1 Building blocks of a metrology program

A metrology program should be composed of multiple elements, including a process for qualifying instruments when purchased or when a component is upgraded, an accurate inventory and tracking system containing information on individual components of the instruments or systems, an effective record keeping system, and a calibration and maintenance program. SOPs should define the program for each step of the process. Personnel with appropriate training for their responsibilities are also part of an adequate metrology program.

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5.1.1 Inventory management

Being able to verify that the instruments and systems are performing accurately at the time of data generation is the key to ensuring data quality. In order to accomplish this, an organization needs to know which instruments and systems they own, and also their historical and current state of calibration and maintenance. The simplest way to do this is to purchase metrology database software. For organizations with only a small number of instruments and systems, the database could be as simple as an Excel spreadsheet. These metrology or Excel databases should contain appropriate data fields, such as system and individual component identification, manufacturer, model, serial number, description, location, and custodian.

In addition, critical performance parameters, such as range, resolution, and user requirements, can help identify the appropriate equipment to be used for specific measurements. Calibration dates/schedules, and also the status of the instrument and components (active, out of tolerance, retired, or awaiting service), can be tracked.

A person responsible for taking inventories and maintaining the database is necessary for an inventory management system to be effective and can be identified in

the SOP. A good metrology database will also provide automatic reminders when the equipment is ready for re-calibration or maintenance.

5.1.2 Instrument qualification

New instruments in the laboratory should be set up and their performance qualified against both the manufacturer's specifications and the purchase criteria.

1. Installation qualification (IQ). IQ demonstrates that the equipment/system has been installed correctly at the user site according to vendor standards. The vendor should install the equipment to demonstrate to the buyer that all the components are operating properly. The qualification process includes appropriate documentation of the system components, physical installation and hook-up, and a performance check to verify that the individual components operate and can communicate with each other. System component information, such as serial numbers, type of use, and user performance requirements, should be included in the metrology database for easy tracking and scheduling of maintenance and/or calibration.

2. Operation qualification (OQ). OQ involves verifying that the system operates according to the specifications as agreed between the vendor and the purchaser. This should include a test of each critical component and function according to the vendor specifications and user requirements (if different) using specific standards. OQ is usually conducted by the vendor; however, in-house or a qualified third-party contractor may be used. The qualification can be conducted on each component of the system or holistically on the entire system. If each component is qualified, then the system has been operationally qualified. However, if one component does not qualify, the system cannot be qualified. It is an all-or-nothing proposition. If the instrument contains computer software, then its ability to capture, store, transfer and manipulate the data accurately should be validated at this time. Detailed test scripts and a complete qualification documentation package need to be generated. Many vendors now supply OQ documentation packages. In-house procedures need to be written to address custom specifications. If a component is replaced or upgraded, a new IQ and OQ need to be conducted on this component. A complete OQ is usually not required for the system.

3. Performance qualification (PQ). PQ and system suitability (SS) demonstrate that the performance specifications of the system meet the user's expectations and needs for a given use. PQ can be a general validation of commonly used parameters or a specific method validation. At a minimum, the PQ should include expected performance and limit/failure testing. The PQ of a method can include demonstration of precision, resolution, separation, recovery, and signal-to-noise ratios. The PQ should be conducted before putting the instrument into routine use. PQ may be repeated many times during the life cycle of the instrument as new methods with different performance criteria are used. Additionally, PQ should be conducted after routine calibration and maintenance, relocation, repair, and component upgrades. The user or a qualified third-party contractor can conduct this qualification. The user performs PQs daily, usually prior to and throughout sample analysis, using specified standards

and performance criteria. Again, the performance of the system is documented so the accuracy of data generated can be verified.

5.1.3 Calibration and maintenance

To facilitate the maintenance of equipment with different performance criteria, written procedures are needed that serve as a record of the process used to evaluate the system's performance. Maintenance by appropriately trained personnel should be performed at regular intervals before equipment parts fail.

Defined maintenance procedures should include model or manufacturer specifics and a list of parts to be inspected, cleaned, lubricated, replaced, and/or calibrated. The replacement part numbers, cleaning solutions and lubricants, and calibration standards, along with the manufacturer's maintenance procedures to be followed, should be specified. Documentation is easily managed by creating a one-page checklist of instructions or performance parameters that can be checked off as each task is completed. Any issues or comments can be captured directly on the checklist. Provisions for failure or out-of-tolerance notification need to be clearly defined, as the equipment cannot be put back into service until the performance has been verified by conducting a performance qualification.

As part of maintenance, some equipment may need to be calibrated. SOPs must include calibration methods, and a report format needs to be available at the time of calibration. Calibration SOPs must include pass/fail specifications as well as corrective actions to be taken in the event of calibration failure. For quality calibration standards use National Institute of Standards and Technology (NIST) traceable standards or other intrinsic standards of known purity, quality, and stability. These standards should have certificates attesting to their performance properties. After calibration, the metrologist or other responsible person should review the calibration reports to identify any issues with the equipment that may need further attention.

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5.1.4 Documentation

An organized document filing system must be maintained. This could be a paper file, an electronic document file, or a mixture of both. The equipment inventory system contains key information on the components of each system, their performance criteria and maintenance and calibration status. All documents including installation and performance documentation, as with other documents necessary to demonstrate the quality of the data, include SOPs for the qualification procedures, calibration, maintenance, personnel training, etc. If the manufacturer's operating, service, or maintenance manuals are used or cited in the operating procedures, copies of these manuals should be maintained. To facilitate retrieval, documentation should be stored in a central location and be indexed for easy retrieval.

5.1.5 Quality assurance

In addition to incorporating the preceding elements into any metrology program, periodic audits of the calibration and maintenance practices should occur. This is particularly important for systems generating data that are subject to review by

regulatory agencies or certifying organizations. Audits should check the thoroughness and completeness of documentation and procedures, as well as adherence to those procedures. Documentation auditing will include qualification, calibration, maintenance, and training records of those responsible for the organization's metrology program. Additionally, adherence to written procedures and a check against current regulatory standards applying to the organization should be conducted. These process audits provide a system of checks and balances to help ensure data quality.

5.1.6 Assuring data quality

An organization interested in assuring data quality from the time of data generation through its life cycle should seriously consider including a metrology program as part of their routine scientific practices. Many elements of such a program may already exist within the organization, such as SOPs, certified reference standards, quality assurance/quality control verification, and calibration and maintenance of instruments. A formalized metrology program will provide a point of control and standardization of processes that could significantly reduce the cost of generating true and accurate data and result in more satisfied customers and more GLP-compliant raw data and study results.

5.1.7 Vendor audit

Before purchasing an electronic field or laboratory data capture system, a vendor audit must be conducted. Table 1 provides a sample check list for evaluating a computer system vendor. The audit team should be represented by the QA unit, an IT services representative, and users to look at system development, change and defect correction procedures, and documentation. Concerns that the vendor is a relatively small company that could 'go away tomorrow' and leave a company with what is sometimes called 'vaporware' should be discussed. Ensure that the vendor will provide their standard lease agreement, which stipulates that in the event they cannot support the software in the manner specified in the agreement, the company would receive the system code for the purpose of maintaining a useable system. Recommendation reports, which include a thorough cost–benefit analysis, are written. Copies of the program are needed for Study Directors, research farm cooperators, sample receipt and processing groups, and field coordinators. Planned limited testing of the program against paper forms is needed.

6 Quality assurance (QA) and data audit

Auditing data that have been captured, manipulated, transferred, and reported electronically has produced new challenges for QA personnel. In general, auditing electronically captured data from studies conducted in compliance with GLP should be approached no differently than performing any other data audit; however, there are additional considerations.

QA personnel who audit and monitor the GLP compliance of computerized systems should be familiar with and/or receive training on each system that is utilized in

Table 1 Computer system provider checklist

COMPANY HISTORY

Item	N/A	Yes	No	Comments
1. How long has this company been in business? 2. How long has this product or version been in production release? 3. How many previous versions have there been? 4. What are their working/support hours? 5. Is financial status provided (annual report)? 6. What percentage of sales is to pharmaceutical companies? 7. Is the provider familiar with appropriate government regulations/ industry standards? 8. Does the provider hold any certifications (e.g., ISO)? When was this achieved? Is it current? 9. Is there a list of previously installed systems available? 10. Is there a list of acceptable software and hardware suppliers?				

FACILITIES

Item	Yes	No	SOP[a]	N/A	Comment
1. Does the computer room have limited access (e.g., are the servers physically/ logically secure)? 2. Are there provisions for power backup? 3. Is there a disaster recovery plan and is it periodically tested? 4. Is there environmental monitoring? 5. Are there off-site back-up facilities for key documents and software? Is it readily retrievable?					

[a] SOP = is there a SOP or is it addressed?

PERSONNEL

1. Are organization charts provided depicting structure and reporting relationships of QA and software/hardware development? 2. Do job descriptions exist? Are there current curricula vitae for all employees that detail education and experience? Are they routinely updated? Are they retained for past employees? 3. Is there a written procedure for training and is it documented? 4. Does the company have a QA department? How is QA provided?					

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Table 1—*Continued*

Item	Yes	No	SOP[a]	N/A	Comment
5. Is there an internal audit program? How is follow up tracked?					
6. What is the ratio of QA to development staff?					
7. Does QA have the authority to reject software programs or hardware?					
8. Are testing records reviewed by QA? Is this documented?					
9. What is the number of staff assigned to perform various functions (developers, managers, administrative)?					
10. What is the average length of employment?					
11. Is there training available for regulatory aspects for the development staff? Is the company familiar with appropriate regulations (GLP, GMP, GCP)[b]?					
12. Is the staff knowledgeable on industry standards and practices?					
13. Has the company ever been inspected? By what organization?					
14. Were there any legal actions or regulatory observations from the observations?					

[b] GMP = good manufacturing practice; GCP = good clinical practice.

STANDARD OPERATING PROCEDURES

1. Are SOPs or other procedural documentation in place for the following: requirements, design programming, testing, source code, configuration management, change control and retirement?					
2. Are there SOPs or other procedural documentation for the computer room: security, back-up/recovery and disaster recovery?					
3. Are relevant and current SOPs available in each area?					
4. Are SOPs signed by management and dated?					
5. Are SOPs periodically reviewed and updated? Is training performed after the updates?					
6. Are deviations from SOPs documented?					
7. Are historical copies of SOPs maintained?					
8. Is there a SOP for archive requirements and retention policies? (Is the archived material indexed, is there document control and is it secure?)					
9. Do the SOPs provide sufficient detail including personnel responsibilities?					
10. Is there a SOP for version release?					

Table 1—*Continued*

SYSTEM DEVELOPMENT

Item	Yes	No	SOP[a]	N/A	Comment
1. Is a structured methodology approach followed? Does it address life cycle characteristics (requirements)?					
2. Are there periodic software development review meetings and are these meetings documented?					
3. Does someone other than the system developer perform final testing?					
4. Is the testing documented?					
5. Are there management approvals for development activities?					
6. Are there programming standards (i.e., version numbering)?					
7. Are there documentation standards?					
8. Are there overwrites, white-outs or pencil entries on official records?					
9. Are there standards for programming naming conventions?					
10. Is source code stored in a restricted/secure location?					
11. What documentation is available/will be supplied for the following:					
a. Functional requirements for each software/hardware module					
b. Detailed design specifications (including a technical diagram of how the system operates – may also include hardware and software configurations, module overview and screen formats)					
c. Listing of error messages					
d. Calculations used (if applicable)					
e. Business rules (e.g., how the provider designs/builds rules in the application)					
f. Environmental requirements, limitations, assumptions and exclusions					
g. Details of the application programs (e.g., language, database, client server)					
h. Description of the database, data model, file design, table structure, interrelationships of the data, field level mapping					
i. Samples of reports					
j. Examples of test data sets available (e.g., test scripts or automated test tools that would be suitable for version purchased)					
k. Boundary/stress/unexpected input tests					
l. Structural/functional testing (documentation of walkthroughs, etc.)					
m. Documented test results, exceptions and acceptance					
n. System constraints					

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Table 1—*Continued*

Item	Yes	No	SOP[a]	N/A	Comment
o. Impact to network (does it run on LAN or WAN?)[c] p. The security aspects of the system/ application q. The audit trail function in the application					

[c] WAN = wide area network.

MAINTENANCE					
1. Is there a program for handling customer complaints, complaint investigations, and corrective actions? 2. Are there management approvals/acceptance for change control? 3. Are there management approvals/acceptance for source code modifications? 4. Are records retained for lifetime of the product/version for support? 5. Does the User Manual represent the current system? Is the manual updated as changes are made? 6. Are notifications of bugs and resolutions available to all clients? When does this occur (e.g., how many days from initial notification?) 7. What is the procedure for documenting a problem? Does it address the following: how, when and by whom it was discovered, how, when it was resolved and who is making the change? 8. Will vendor perform installation qualification and provide the documentation for this? 9. Are test errors monitored for trending purposes? 10. Generally, what is the average number of bugs found in new versions of software within the first 30 days of release? 11. Will a statement regarding archival of source code and access in the event of a request from a regulatory agency be prepared and signed (i.e., escrow agreement or equivalent) 12. Are electronic/hard copies maintained for all versions of software and documentation? 13. Is there an estimated timetable for version updates? 14. Are older versions of software upward compatible with newer versions? 15. How are versions retired? 16. How many versions of software are supported simultaneously? 17. How are new releases or updates (patches) conveyed to clients?					

electronic data capture and handling for the data they are to audit. While the training of GLP study personnel is extremely important, management cannot overlook the fact that QA auditors must also receive training. Without a thorough knowledge of software operation, QA cannot adequately audit electronic data. This data capture training could include hands-on training, attending outside training courses, and reading available documents about the system (i.e., the validation report, applicable SOPs, system user manuals). Additionally, the auditor should have training in the regulatory areas related to computer systems. In some companies, there are QA personnel with specialized training within the QA unit who are assigned to audit computerized systems. In smaller companies with limited resources, this specialization may not be practical.

GLP regulations require QA personnel to inspect/audit each study conducted, but the extent to which QA personnel are involved in software development and the validation/verification process varies from company to company. In some companies, there is little or no QA involvement in these processes, whereas in others QA personnel are involved. QA personnel can provide assistance in the area of vendor audits for purchased software or can conduct inspections of in-house software development to ensure that internal procedures are being followed. QA personnel, who conduct in-process inspections and review the resulting data and validation report for accuracy, could provide inspection support during the validation and verification process. During system development and validation, properly trained QA personnel can provide the regulatory advice needed to ensure that the system will meet government standards. QA personnel become more familiar with the system(s) that will be used when they are involved early in the validation process.

The QA unit should have written procedures (SOPs) for the conduct of inspections and audits. These procedures should incorporate all considerations for the review of electronic data systems. The QA unit SOPs should address the role and responsibilities of the QA unit in software development, purchase, and validation activities, in-process audit procedures for data collected on line, procedures for on-line review of data (i.e., what will be verified and how much data will be reviewed), and the procedure for auditing reports using on-line data.

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6.1 Critical areas to consider for auditing field studies

Many of the software programs used in field studies perform calibration/application calculations and the calculation process is validated/verified. However, QA should conduct an independent check of the calculations used with the program and the data for an added comfort level.

In electronic data packages used in field studies, there are some electronic data that are directly entered by the field investigator (FI) and some that are generated by the program (e.g., dates and times). Both types of raw data need to be verified by QA personnel, who should check not only directly entered data, but also computer-entered data. In order to conduct a thorough data audit, QA should determine which data are direct entry and which are automatic.

QA should ensure that notes and various descriptions (e.g., sampling method, test system observations, etc.) are clear and thorough during the raw data audit. In addition, all audit trails should be checked for clarity and to ensure that each

original entry can be determined as required by the GLPs. QA must be able to ensure that all data entries 'make sense', especially in connection with event times and dates.

With electronic notebook studies, there will also be paper data to audit. This will include facility data (e.g., weather data, equipment maintenance records, storage temperature logs, personnel records, etc.) and study specific documents, such as faxes, e-mails, paper notes, etc. When paper data have been transcribed into the electronic notebook, they should be checked by QA to ensure accuracy. Any data that have been transcribed for whatever reason must be identified as such with the original raw data attached to them. QA should ensure that all paper data have adequate identification (e.g., study and trial numbers), that they are recorded per GLP, including dated signatures, and that all of the pertinent paper data or exact copies are sent to the Study Director for archiving.

6.1.1 Preparation

In order to audit effectively studies with electronically generated data, QA should create a special checklist or add electronic issues to a current checklist. The checklist should include items such as ensuring that the computer system in use is current and validated and that necessary maintenance is documented.

Prior to conducting audits, QA should review the protocol plus any amendments and deviations. QA should ensure that the paper protocol and any changes to it will be readily available during study events in case the documents need to be referenced for information. Protocol amendments should be available electronically if the system allows and this transfer, if necessary, should be checked by QA. Any previously recorded electronic data should be reviewed in advance if possible. Prior to going into the field, QA should print blank 'forms' because following the screens during electronic data entry may be difficult. There are two main electronic field data collection programs used in the USA for field studies: FieldNotes and *i*-Advantage. Occasional reference will be made to them throughout this section to describe a QA procedure. For *i*-Advantage, blank forms may be generated directly from the program, and for FieldNotes, blank forms can be downloaded from the Astrix Web site. Having printed blank forms available during the field trial is also a good idea for the FI for backup in case there is a problem with the electronic system. QA must ensure that backup electronic data copies are being made as required by protocol and/or SOP and that any required incremental updates are being sent to the Study Director within the allowed time frame.

6.1.2 Paper vs electronic data audit

Some QA auditors prefer to audit printouts of the electronic data rather than the electronic data themselves. This may be partially due to a lack of familiarity with electronic auditing or with the computer system and partially to unavailability of the software to QA. There are several arguments in favor of QA auditing the electronic data rather than the paper data. For one, there may be perceived compliance issues related to the printouts that do not exist in the electronic data (such as no initials and date on the hard copy). Also, QA may not be given printouts of all the pertinent data,

such as site logs. If QA does choose to audit the printouts of the electronic data and the site logs, audit trails and notes pages must also be printed and audited.

In order to audit electronic data, QA must either own their own copy of the software or use the computer where the software is installed, the 'community computer'. In the case of FieldNotes, the use of the 'community computer' may be more practical since QA would have to purchase the program for its own use. When using the *i*-Advantage program, cost is not an issue, as the program is available without charge to any QA auditor, so resources should not be an issue. When auditing electronic data, following the whole study may be difficult since documentation cannot be spread out on a desk. For this reason, it is recommended that certain pages, such as the audit trail and notes pages, etc., be printed out for easy reference.

FieldNotes user verification can be conducted by following the SOP written by Astrix, which can be downloaded from the Astrix Web site. The SOP contains a script that is typed into the computer system. If the printout matches the SOP script, then verification is complete. A record of this must be placed in the facility archive. This verification process takes 1 h or less. For FieldNotes, calculations are not verified at the field site since the manufacturer feels that the software developer and the sponsors have performed adequate validation of these calculations previously. This may, therefore, require a visit to the sponsor or to the manufacturer to confirm that this important step of the validation process has been completed and is adequately documented. By following the Astrix SOP, the user is simply ensuring that the program operates on their system(s). The SOP script must be typed in exactly as written in order to confirm the printout accuracy. QA must ensure that the verification documentation is properly archived. Just as with any other SOP, this SOP must be approved in writing by field site management to comply with GLP.

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For *i*-Advantage, more extensive field site verification is conducted. A field site notebook is used which verifies every step in the data entry process. Following this procedure, a form is completed and returned to American Agricultural Services, Inc. (AASI), where the form is checked to ensure that verification was properly conducted and documented. This verification takes approximately 2 h to perform. Documentation and verification may require a visit to AASI to confirm that the validation process has been completed and is adequately documented.

6.1.3 Late-entered data

In any situations where data need to be entered late, which will result in data that appear unusual, a thorough explanation is needed. If, for instance, the laptop battery dies and there is no adapter available and only part of a calibration was completed before the laptop shut off, there will be some manually recorded paper raw data, which the FI will have to transcribe later into the computer data system. It is important to realize that when this happens, the paper data generated become the raw data for that portion of the study, and the electronic record is the raw data for the earlier portions of the study. Since the program time stamps data, some data will have atypical entry times. These need to be explained to prevent the perception of 'creating data in the office'. The explanation can be done simply by attaching a note to the transcribed data indicating the situation and referencing the source of the transcribed data. In this case, the original paper raw data would need to be retained with the other study raw data.

6.1.4 Test substance tracking

For FieldNotes, the general log for the test substance will automatically track usage for each application at all sites. When the same container of test substance is used for several studies, the data will appear to be atypical. If QA is auditing individual study data, the numbers will not seem to be accurate for a particular study. QA must be made aware when one container is used for different studies during the audit. Consequently, sending a separate container of test substance for each trial and/or study is recommended in order to facilitate tracking during the audit.

6.1.5 Generic menu choices

Many of the entries in both FieldNotes and *i*-Advantage are made using drop-down menus. The choices are not always the most accurate for the situation at the field site (i.e., sprayer types, nozzles, crop stage). QA should check during the raw data audit to ensure that the choices made are an accurate reflection of the actual practices and equipment that were used. The FI may need to attach a note to the pertinent page to clearly explain certain situations, such as the use of unique nozzles or the use of different-sized nozzles for air blast applications. Additional explanations may be required for air blast applications. Owing to the variety of air blast calibration procedures utilized by field sites, the electronic forms may not correspond to the way in which the units are calibrated at the site. For example, if the form only allows for entry of total calibration output volume over a given time, but the FI collects individual nozzle outputs over a timed period, this would need to be indicated in an attached note that includes data for the individual nozzle outputs with the appropriate units of measure. This situation may also occur when an in-line flow meter is used for sprayer calibrations, where the initial volume is not typically noted. If the electronic data form requires entry of initial volume, this entry can be given as an estimate with a note attached explaining the situation.

6.1.6 Computer power considerations

The battery life in some laptop computers is a consideration. Some laptop batteries may last long enough to perform calibration and application; some may not. Additionally, there may be unforeseen circumstances that may require longer access to the laptop or that may cause premature battery failure. In order to ensure that data are not lost, a source of backup power, such as a spare battery or an adapter that plugs into the vehicle cigarette lighter, should be available.

6.1.7 Backing up and transferring data

Systems for backing up data are required. There are a variety of media that will meet the requirement. The decision should be based on logistics, resources, and systems already in place. Some appropriate back-up media are floppy disks, zip drives, additional hard drives, network server and CD-ROMs. Data must be backed up at least once per day on the day that original raw data are entered into the program. Ideally, backed-up data should be stored in a fire-resistant, secure area or a secure location separate from where the primary data are stored.

When study data need to be transferred to the Study Director at regular intervals (typically after each application, sampling, and shipment), the e-mail and attachment transfer rate is critical. In isolated field sites, with poor telephone line quality, the upload can take hours. This is also a problem when downloading software updates. Also, the upload/download time may be a problem when it ties up the only telephone line or fax line. If available in the area, the use of a faster form, such as cable modem or digital subscriber line (DSL), is preferable. Since telephone line quality is a common problem, it would be best if software developers sent updates on CD-ROMs.

6.1.8 Hardware and software security

For the security of the hardware, the laptop should be kept in a secure location, as secure as a paper notebook would be kept, whether in the field or in the office. In addition, the storage environmental conditions are particularly important and temperature extremes and high humidity must be avoided.

The use of individual passwords increases software security. During a study inspection, QA should ensure that the person physically entering the data is the person that has logged on to the system. If not, this is a GLP compliance issue since the person entering the data will not be the person identified as such in the electronic data. QA personnel should have 'read-only' access to the data in order to avoid any inadvertent changes.

6.2 Critical areas to consider for auditing analytical laboratory studies

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In addition to observing the procedure being performed during the conduct of in-process inspections of chemistry analyses, the data collection practices and the data capture system should be inspected. The QA auditor should review the protocol and applicable SOPs for data collection practices and the procedure prior to conducting the in-process inspection.

The auditor can typically observe several items related to data collection practices during in-process inspections. Just as during any other in-process inspection, the auditor should observe if the protocol and the applicable SOPs are being followed with respect to the procedure and data collection practices, if the appropriate security procedures are being utilized, and if changes are documented appropriately (i.e., the original entry is available, the reason for the change is documented, etc.). Additional items to check include computers left unattended without the user logging out, user name and password posted in the laboratory, and group use of user name and password. These are all GLP compliance issues, and they are probably also SOP deviations.

Additional items that can be reviewed during the in-process inspection include personnel training records and equipment records. The personnel training records should be checked to verify that training on the computer system has been documented and that the individual(s) observed during the inspection has the appropriate training in the technique. Equipment records should be reviewed for the equipment used during the in-process inspection or they may be reviewed during a facility inspection. These records generally include documentation of maintenance and, when applicable, calibration. Each computerized data acquisition system should have maintenance

records. Maintenance for a computer system should include both hardware and software. If a controlled change was made, such as a software upgrade, the records should indicate what was changed, why the change was made, who made the change, when the change was made, and if the system was revalidated after the change. An authorization procedure should be in place and included in a SOP for approving controlled changes. This authorization should be documented.

Unanticipated events that occur should be recorded in computer system maintenance records and include what corrective action was taken, who performed the corrective action, and when the action occurred. These events might include system crashes, date/time changes after a power failure, etc.

6.2.1 Data/report audits

QA must review the final study report to make sure that the report accurately describes the methods and SOPs, and that the report accurately reflects the raw data generated during the study. QA personnel need direct access to the on-line data to adequately perform a data/report audit. Access for QA personnel must be in the form of read-only access.

QA SOPs should specify the amount of data to be audited and how the data points are chosen for audit. An auditor may choose to perform more thorough and more frequent audits on a recently validated system. The validation report can be used to assist in determining what and how much to audit. For example, if data summary printouts from the chromatographic computer system are used in the report, the validation report should be reviewed to verify that this summary function was tested during validation. If this portion of the computer software was successfully validated, verifying a few values from each table in the report may be sufficient.

QA personnel should review data on-line. Data changes need to be reviewed to ensure that the audit trail was appropriately generated and maintained (e.g., the original and changed data are both available, the date of the change was recorded, the reason for the change was recorded, and the person responsible for the change was identified). No data can be overwritten. For example, reintegrated or recalculated data must not overwrite the original data. The on-line data review should also include the tracking of several samples through the system and a check of the calibration and integration parameters. If data were reintegrated, all integration parameters should be saved and audited. If spreadsheets or statistical packages are used, these data and analyses should be included in the audit. Items such as input values and equations or routines used need to be verified during the data audit. This type of software should be included in the validation program to ensure that the software is providing the correct output values.

6.2.2 Maintenance and repair records

Although computer equipment is used differently to other field equipment, raw data are being generated; consequently, maintenance records are required. All maintenance and repairs to the computer system need to be recorded. For example, software and hardware maintenance records would include system/program updates, disk scans, and defragmentation (e.g., preventative maintenance). Repair records might include communication with the software developer about problems/bugs and steps taken to fix any software or hardware problems. Documentation must include whether the

procedure was routine and followed SOP. Documentation of unscheduled repairs must include the nature of the problem, how and when the problem was discovered, who discovered it, and what corrective action was taken to comply with the GLP requirements.

6.2.3 Records retention

The responsibility of the QA unit should not be limited to in-process inspections and data and report audits. To be in full compliance with GLP, the QA unit should review all procedures for storing and archiving electronic as well as paper raw data. This review should include ensuring that back up and archiving procedures were performed as specified in the SOPs, that archiving was documented properly, and that long-term storage procedures were followed.

Archived electronic data are to be treated no differently to archived paper data. An archivist should be assigned, access should be limited to authorized personnel, data should be archived at the completion of the study, and all material should be indexed to permit expedient retrieval. Depending on the medium used for storage, an area within the facility may be needed with specific environmental controls to maintain the integrity of electronic data. This should be specified in the data storage SOPs. Environmental conditions need to be monitored in the archives where electronic media are stored.

6.2.4 Facility inspections

Computerized systems should be included in facility inspections of field sites and analytical laboratories. Items discussed previously, such as computer maintenance records and personnel training records, can be reviewed more thoroughly during the facility inspection.

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Training records should be reviewed for all staff involved in software development, validation, and computer maintenance in addition to the personnel training records for field and laboratory technical staff. The computer personnel may reside in a separate functional group or may be part of the laboratory group. In any case, training records must contain documentation of education, experience and training to support the duties that they perform. Depending on the function performed, this training should include training in GLPs and other regulations or guidance documents.

The facility inspection should include a review of the computer systems and software to ensure they have been validated. There may be differences between how network systems are validated in contrast to stand-alone systems. The records, procedures, and SOPs for the different systems should be reviewed as part of the facility inspection. If validation reports have not previously been reviewed by the QA unit, the facility inspection may provide an opportunity to review these reports and data.

Each computer system used to capture data during GLP studies needs to have records of maintenance. These records must be reviewed to ensure they are compliant with the applicable SOPs and GLPs. Consistency of record keeping between systems should also be reviewed. Items such as system to system variation in time/date settings, passwords being changed as required, completeness of software documentation, hardware upgrades for each system, availability of maintenance records, user manuals, and other system documentation in the laboratory, can be evaluated during the facility inspection.

6.2.5 Validation and user verification

Under the GLPs, all systems generating data electronically must be validated. In the case of software currently in place, this validation is conducted in steps. First, the software developer thoroughly validates the program prior to its release. Sponsor companies perform additional validation using their own protocols. The field site user must verify that the program works on the system(s) utilized at their site. Each field site user must have written SOPs in place approved by management that detail how this will be done at the field site. This verification must be done prior to conducting any field studies with the system and following each software change or update or significant hardware change. QA should check to ensure that all systems have been adequately verified for the most current software system in use and that each verification has been properly documented. Current system specifications can be obtained from the software developer. QA must ensure that the verification documentation is properly archived.

7 Validation of computerized systems

Many quality programs and company standards require the validation of computerized systems. Systems that generate or manipulate data must be validated to fulfill various regulatory requirements. Failure to perform adequate validation may result in a lack of confidence in the data generated and regulatory noncompliance.

For computer systems that do not require formal validation, at a minimum, user acceptance testing should be performed. This User Acceptance Testing should be outlined in a facility SOP, where the system is tested and assured to perform in the desired function effectively. The User Acceptance should be documented, and the system should be released for specific uses.

The FDA defines validation as 'establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes'. Various approaches may be used to fulfill this requirement.

Computer system and software validation may be regarded as a series of steps taken to determine whether computer systems are able to meet the demands placed on them, including functionality and reliability in a production environment. Software validation can increase the usability and reliability of a system, and can reduce failure rates. Software validation includes all the testing activities included throughout the software life cycle. Validation should begin with software design, will include planning, execution, analysis, and documentation of appropriate validation activities and tasks, and will continue throughout the life of the software.

Software and computer systems that are subject to validation must be designed using strict procedures with sufficient documentation. During the process of system design, strict controls must be in place to allow future validation success. The system designer must ensure that documentation of the system meets minimum requirements necessary to satisfy the needs of the validation team.

The following steps should be formalized within a facility through written SOPs. These SOPs are to be approved by management, and their purpose is to ensure that appropriate personnel follow the procedures judiciously. QA personnel will be

involved in the process as outlined in facility SOPs but will minimally review operations for adherence to SOPs and verify that documentation is adequate and appropriate. QA will report their findings to management, per SOP.

7.1 System life cycle

7.1.1 Needs assessment

The first step should be an assessment phase: to identify and define needs based upon business goals and target users. All basic functions of the software or system required by the user, including primary inputs, reports, and calculations, should be identified. The regulatory impact, including data integrity and security issues, should be listed. The vendor's history (as applicable), the purpose of the software application, the volume of data that will be collected by the system, the effect that the new system will have on existing systems, applications, and procedures, and the use within departments must be considered. A project management plan, outlining timelines, deliverables, and specific tasks should be drafted. Management must consider all information and make the decision whether to authorize development or procurement.

7.1.2 Requirement definition

Upon management's approval, the end-user requirements for the software/computer system should be identified in a formal document that includes the following:

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- an introduction summarizing the purpose
- a general description of the system including hardware, interfaces to other peripherals, audit functions, control functions, communications, protocols, safety, and security considerations
- all inputs and outputs of the system
- performance requirements
- error handling.

The document should be reviewed for correctness and completeness and audited by the QA unit.

7.1.3 System providers/vendors

At this point, the availability of a purchased product that fulfills the software/computer system design description should be researched. If a commercial product is not available, and the capabilities for software/computer system design are available in-house, the design phase can be initiated.

7.1.4 Design

The programming staff or IT department personnel should develop a software/computer system design description based upon the requirements document. This description should outline the specifics required in the system, including security

measures, criteria for acceptance, information flow, data handling, etc. A formal design review should be conducted by the appropriate personnel to verify that the software/computer system design description is adequate and correct.

7.1.5 Development

The detailed design description is translated into source code. Useful descriptions for a module, including expected inputs and outputs, operations to be performed, and expected data types, are often provided in code comments. Source code evaluations, such as code inspections and code walkthroughs, should be conducted to verify compliance with the corresponding design specifications.

Crucial documentation needed in the development phase includes end-user manuals, a unit test summary report, a user acceptance test plan, results of the database design, results and methods employed in the source code evaluations, and a traceability analysis. A source code traceability analysis verifies that all code is linked to established specifications and established test procedures.

Test plans are drafted that will include a description of all tests to be run, the purpose of each test, the data sets to be used, the identification of the input, and the expected output.

7.1.6 Testing

This is a critical phase. Testing includes demonstration of compliance with all software specifications and production of evidence that attests to the fact that defects which may lead to errors or problems have been identified, remedied, or removed.

Testing includes strategies to find software defects and tests designed to prove that the software works. The strategies and tests will provide different results.

The test plan drafted during the development stage should provide a thorough method for evaluation of these elements: system security, data storage, data retrieval, audit trails, data integrity, measurement accuracy and reproducibility, stress testing designed to identify sources of system failure, assessment of reporting formats, and traceability.

Test plans should include definite, measurable acceptance criteria, in addition to the amount of testing to be done. The test plan should include the data sets to be used and the detailed instructions for testing. Errors encountered during testing must be documented, including how they were discovered, their description, and any action taken to remedy the error. Errors must be remedied prior to the release of the software or computer system. Test results must be documented clearly to allow for pass/fail determinations to be made.

Testing must be conducted in a typical end-user environment, or in a simulated end-user environment, identical with the environment where the software/computer system will be used. Documentation of testing can be recorded as raw data, such as in a logbook, and should include the parameters tested, and the results of testing. The data should be tabulated as a final report document that includes all details included in the test plan, their execution, the results, and conclusions. The final report document must be signed by appropriate personnel, reviewed as needed, and archived. Upon successful completion of testing, the software/computer system can be released for testing and use in an actual end-user environment.

7.1.7 End-user validation

Software producers must fulfill the requirements for development, testing, and documentation. When the user is confident that these requirements have been met, and the system functions adequately to fulfill his or her needs, the user must perform validation prior to placing the software in service. An analytical laboratory is an example of an end-user that conducts testing prior to software/computer system installation.

7.2 Validation of chromatography software

Because chromatography acquisition and processing software is complex, exhaustive end-user validation is impractical. With assurance and documentation that the vendor and developer have performed the required steps and testing, a focused end-user validation can be conducted. A more manageable alternative to testing every feature of this complex application is to prove that the system meets specific user requirements. Necessary data collection, processing and reporting can be tested, while software features extraneous to a facility can be omitted. This is a more efficient approach, but this selective testing requires users and managers to correlate software features with laboratory operations and requirements.

End-user software acceptability testing is frequently performed to satisfy regulatory requirements; however, it is also used to ensure system validity and identify deficiencies.

Frequently, software is tested by a facility by 'contriving' a situation where the software will likely fail, then running the software and monitoring its failure. This testing is often duplicative of the type of testing conducted by the software architects. A more reasonable facility, end-user test, is to use the software in its intended application, testing it in its actual function. While this approach is somewhat limited in scope, the functionality of the software in its working environment is tested directly. This testing, however, must be thorough and well mapped out. Keep in mind that each application of the software must be tested. This can limit the flexibility and expandability of the software for novel uses, as testing must be done for each use. Adequate documentation must be completed and archived prior to each installation.

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7.3 Validation priority setting and risk assessment

After SOPs have been written for the use, maintenance, and acceptability criteria for the use of the software/computer system, individual users have been fully trained and the training has been documented, the software/computer system has been fully installed, and the end-user has been validated, the software is ready for use.

Any changes or additions to existing software are considered design changes and must be subject to design change control provisions. Validation procedures associated with each software change must be documented in a logbook as part of the record of that change. All changes regarding operation of validated software/computer systems need to be fully documented, including errors, change control logs and qualification logs, and need to comply with the written SOPs. This quality control system must be maintained for the life of the software/computer system.

Because the validation of computerized systems is time consuming, expensive and resource intensive, many organizations are challenged to identify and prioritize which systems will be validated. There are organizational and system specific risk factors to consider in the regulated environment. Each organization must establish its own risk assessment process.

Regulated organizations are expected to be able to demonstrate to government inspectors that they have control of all processes and systems that affect data integrity and quality. The organization's validation priority setting decision-making process should be documented, on both organizational and system-specific levels. Ongoing maintenance and validation verification logs must be up-to-date and readily available for inspection by QA or other auditors as the need arises for system validation verification.

7.4 Organizational considerations

The following considerations must be addressed when validating computerized systems:

1. Define what computer system validation would ultimately entail in a management-approved document, such as a policy statement.
2. Develop policy-supporting SOPs on computer systems. These may include development, testing, maintenance and support, quality assurance, change control, source code management, system retirement, retrospective evaluation, evaluation of vendor-supplied systems, etc.
3. Generate and maintain an inventory of all systems utilized by the organization, categorizing them as regulated and nonregulated systems. Identify prospective validation or retrospective evaluation needs for each system and record the current validation status.
4. Determine the risk factors associated with each system if the system should fail. These risk factors should include the regulatory impact, safety concerns, and business concerns (cost, time, and human resources).
5. Regulatory impact includes regulated data integrity, security, and product quality focus. Consider the current regulatory expectation for validating such a system. If the system impacts regulated data or is used to assist in making regulatory decisions, computer system validation is a regulatory requirement.
6. Safety concerns include consumer safety and environmental hazards.
7. Business concerns include company reliance on the system, the establishment of contingency plans, and protection of assets.
8. Additional factors to consider include complexity of hardware, application and system software configuration management, detail of the change control documentation, in-house manpower, increased regulatory inspector's awareness, new and pending regulations, and related regulatory guidelines, i.e., 21 CFR Part 11.
9. Identify systems that require validation based on all applicable risk factors. Document the assessment process and the prioritization sequence that will be followed to address these systems and include justifications for systems that do not require validation.

Some organizations develop risk assessment systems that first determine the regulatory impact of each computer system by using the following questions. If there is no regulatory impact, no validation activities are required by the regulating agencies. However, those systems can be validated for business or other reasons.

1. Does the application or system directly control, record for use, or monitor product quality, laboratory testing, or clinical data?
2. Does the application or system affect regulatory submission/registration?
3. Does the application or system perform calculations/algorithms that will support a regulatory submission/registration?
4. Is the application or system an integral part of the equipment/instrumentation used in testing, release, and/or distribution of the product/samples?
5. Does the application or system define materials (i.e., raw materials, packaging components, formulations, etc.) to be used?
6. Can the application or system be used for product/sample recall, reconciliation, stock tracing, product history, or product-related customer complaints?
7. Will data from the application or system be used to support QC product release?
8. Does the application or system deal with coding of materials, formulated products or package components (i.e., labels and label identification)?
9. Does the application or system hold or manipulate stock information, stock status, location, or shelf life?
10. Does the application or system handle data that could impact product purity, strength, efficacy identity, status, or location?
11. Does the application or system employ electronic signature capabilities and/or provide the sole record of the signature on a document subject to review by a regulatory agency?
12. Is the application or system used to automate a manual QC check of data subject to review by a regulatory agency?
13. Does the application or system create, update, or store data prior to transferring them to an existing validated system?
14. Is the application or system the official, auditable archive or record of any regulated activity?

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For systems with regulatory impact, a numerical exercise is then conducted to prioritize the validation activities. This prioritization considers all of the following: system criticality, industry distribution of the software, regulatory experience, and the vulnerability at downtime. The numerical rating facilitates prioritization of validation efforts utilizing limited resources. The numerical priority ranking of each system with regulatory impact can then be compared with other systems to identify the order in which to address the various activities.

The results of the risk assessment prioritization can be reflected in an organizational validation master plan. Therefore, an organization can demonstrate the sequence in which they intend to address validation activities from a scheduling point of view.

After determining that a system must be validated, setting the validation strategy for that system should include the following:

1. Document the functions that are critical and noncritical in the system based on the assessment.

2. For existing systems, conduct a retrospective evaluation of historical versions. Document the adequacy of the historical documented evidence (historical and current versions) and any actions that will be taken to prove the validation status of the system.
3. Where needed, document the testing strategy that should be conducted for each system function.
4. Validate the current version of the system according to a formal validation test plan.
5. Ensure that the management level approvals have been secured for the appropriate validation documents.

7.5 Validation of in-house and vendor-supplied systems

Before deciding to develop a system in-house, the following points must be factored into the risk assessment process:

1. The amount of in-house resources needed for system development, testing, and validation.
2. The regulatory knowledge and training of the system development staff.
3. The extent of software development, SOPs, or standards already in place.
4. The required level of user involvement.
5. The required level of experience with the targeted development tools.

Risk is not minimized because applications are vendor supplied. The level of understanding and incorporation of regulatory expectations varies among vendors. An assessment of the vendor's quality practices should be conducted.

Regardless of the regulatory area, the overall expectation is that organizations have determined the risks associated with the computerized systems utilized in their regulatory environments and that they have documented evidence of their efforts to minimize those risks and meet regulatory requirements.

8 Electronic archiving

The volume of scientific data being generated today is growing at an ever-accelerating rate. There is a desire, need, and requirement to collect and maintain these data in a readily accessible and tamper-proof way that also ensures a high degree of integrity over an indeterminate number of years. Changing at an equally accelerating rate is the technology used to collect, store, and retrieve these data.

History is full of examples of human beings trying to preserve data and information for future generations. The ancient libraries at Alexandria, Dead Sea scrolls, oral story telling, paintings on cave walls, stained glass windows of the Middle Ages, monastic scriptoriums, and the national archives of governments around the world are evidence of this need. Each example is different enough to demonstrate the problems inherent in the methods of archiving used, be it in languages no longer spoken, transcription errors, media that are fragile, or media that are not portable. They demonstrate that archiving information cannot be a one-time event for a given set of data, but must be a process that needs to be managed for the length of time the data and supporting documents are believed to have value. Failure to set up procedures will result in their loss.

Issues that appear to be very different, yet are quite similar, exist in the electronic age. The IT world has had a tremendously positive impact on science and the business world. The ease of electronic data creation and collection has opened ways to model and solve complex problems to a level never before imagined. The possibilities of IT capabilities continue to grow and expand. Computer use is pervasive in most things we do, but is not, however, without challenges. Challenges of data preservation and the accessibility of data and supporting documents for future generations abound. Software and hardware quickly become obsolete. New software and hardware and new versions of existing products are released regularly. People and organizations rush to embrace the promise of new applications, ease of use, and speedier performance, often giving little thought to the data stored in the current systems. The persistent reality is that technology changes will always be with us. A process to manage changes is necessary for maintaining a high degree of integrity of data and for possible legal defensibility.

A mature IT life cycle management program, a strong records management program, and an organization committed to the principles of these programs are three strategic components of a successful electronic data archiving process. All these components must work in concert with one another.

A well-developed records management program that defines rules by which records and documents are handled from creation to retirement is necessary for a successful electronic data archiving process. In a GLP-compliant system, records are classified by type, such as study-specific raw data, reports, personnel records, etc. Each record type has a defined retention time.

A well-developed IT life cycle management program is necessary to an electronic data archiving process. The rules governing the four life cycle phases must be defined. These phases include the introduction of new technology, mainstreaming technology, containing technology, and retiring technology. When retiring technology, decisions must be made about what data and functionality will continue to migrate forward. This decision should be based, in part, on the rules set out in the records management program. This process presents a periodic opportunity to reassess the enduring nature of the data stored in the retiring technology.

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Those devices that generate, store, transmit, or render data must be a defined part of the IT life cycle management for research and development (R&D) and manufacturing. Laboratory technology, although not traditionally thought of as a component of IT, needs to be included in the life cycle. Analytical instruments, today, are fully IT enabled. They have PC controllers, processors, and data storage devices. They have network access and on-board software for collection, reduction, and rendering of data.

Often, the IT life cycle planning today is cost-, project-, reaction-, or necessity-based rather than being based on a well-maintained master plan. This is not necessarily bad. Projects are sponsored by the local department. Hence project teams are closer to where the needs and record keeping rules are defined. However, the project teams need to understand the technology directions of the larger organization to ensure that the proper infrastructure is in place to support the production system.

Every time technology transitions, a different set of capabilities and limitations is offered. For instance, software may be available on one platform but not another. Each technology change requires careful planning and project management to ensure that there is no disruption to the organization and no loss of data.

A physical component of IT life cycle management is storage media management. Tapes, disks, and other electronic media degrade over time. Optimally, they are refreshed every 10 years. Ideally, this is part of the SOPs for the data centers and archive facilities.

Finally, an electronic data archiving process can only be successful in a committed organization with ethical individuals supporting records management and IT life cycle management programs. Non-IT personnel need to assume stewardship roles over the data, information, and knowledge generated by their organizations. Senior management must support the enforcement of these programs and the role of QA, and also understand the need to track evolving government regulations in the area of record management. Ultimately, electronic data and other supporting information can be the most valuable of organizational assets. Documents and data required for regulatory or patent purposes are often given much attention. Different organizational requirements will dictate the rules on accessibility (the ability to locate and use data, etc.) in an organization.

Availability is an aspect of accessibility. An organization may need anytime- and anywhere-access to data and documentation. Sometimes, speed of access to historic data is a requirement. Recall times can vary. They may be hours, days, weeks, etc. The requirements will most likely depend on the type of data being requested. Also, there are certain cost implications depending on the requirements. On-line storage is most convenient but as data repositories grow, system performance may become bogged down. Hardware and software must be scalable to accommodate potential growth. Data centers charge a premium for ready access. Storing data on tapes or CDs is often less costly but carries the latency of having to retrieve and load data. Remember that off-line also may mean off-site. Records with enduring value and a need to be preserved and readily available must be identified. Their ease of access must be planned for in advance rather than later reacting to technological change. Disaster recovery plans and appropriate levels of system redundancy must also address accessibility of the data.

8.1 Managing durability

Storage media age: physical media must be refreshed approximately every 10 years. Storage media options also change with time. Previously popular floppy disks such as 8-in and 5.25-in diskettes are hard to come by. Similarly, magnetic tapes and tape drives change, thus requiring the transfer of stored information to new media types. One could maintain outdated equipment; however, this simply delays the inevitable need to migrate. Maintaining old storage equipment can be just as expensive as migrating to new media and new equipment. Parts and service for older systems become scarce and expensive. The procedures used to transfer data and other electronic information from medium to medium or medium type to new medium type must be validated and verified by QA to ensure accuracy and reliability in the new copy. Backup and recovery procedures may become obsolete and need to change over time.

Just as storage media age, so does the software used to store and access the data. Software versions become out of date and are neither serviced nor supported by the

vendor. Finding people with the skills necessary to support the software usually is not feasible because they are generally expensive and become more difficult to locate as time goes on. The need to migrate to new versions is a necessity. The new software version and the migration plan must be validated and verified by QA, from both data preservation and functional need perspectives.

8.2 Managing usability

The electronic signal produced as output from IT-enabled instruments is considered to be the raw data. Paper reproductions of these signals are considered to be copies of these data.

Migrating only the 'raw' data – the characters, numbers, bits, and bytes – forward is not enough to ensure usability. The meta data and the context for the application or database must also be migrated forward. Meta data are the code to the machine-stored bits and bytes. Meta data are the data about the data. They describe the data in the database. The meta data documentation describes the method of data capture, the application used to access the data, security rules for the tables and columns, and other descriptive and procedural information. For derived or calculated data, the algorithm or protocol that was used must be known. The documentation then becomes something else that must be preserved. Without the meta data, the reader will only see a series of alphabetic characters. Without the entire described context associated with the data, the data have no meaning.

Conversion rules must be defined and documented. This is best achieved with a management-approved SOP that describes both the IT organization and the functional groups who are responsible for the data. If there are data quality problems, they must be addressed prior to archiving and migrating. When retrieving data in the future, addressing and correcting data quality issues at that time may be difficult, if not impossible. If the data and supporting documents have enduring value, then the quality must be kept high throughout their retention period. The act of archiving, by itself, will not improve data quality.

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8.3 Open and closed systems

An open system is an environment in which system access is not controlled by people who are responsible for the content of electronic records that are on the system. A closed system, on the other hand, is an environment in which system access is controlled by IT personnel responsible for the content of electronic records.

All aspects, including application, operating system, network hardware, etc., must be considered in determining the nature of electronic systems. As indicated above, the main difference between an open and a closed system is simply access. If you have a chromatography data system that operates within your department, the system is closed. The system is closed even if the IT Department runs the server and maintains the network. The system remains closed even if you outsource the IT support to a third-party provider, provided no other company's work interferes with yours.

When you start working across the Internet, the chromatography data system becomes an open system and the FDA rule requires controls. Using FDA's definition of electronic records, the laboratory chromatography data system generates electronic records. Based upon the definition, laboratories will need to consider more than just the raw data files. One must also include the method files, run sequence files, and the integration parameters used for the data analysis. The need for a comprehensive audit trail is a critical component of the FDA regulations. The audit trail is an electronic record and is subject to the same controls.

An electronic record must consist of two components: a human-readable section and a machine-readable (computer) section. The content of the human-readable section will include information about the creation and any additional processing of the data.

8.4 Electronic records and electronic signatures

Adequate controls must be in place to ensure that an electronic signature is irrefutably bound to a responsible individual or business. For signed documents that are maintained but not submitted to the agency, EPA's CROMERRR allows for electronic records to be used in lieu of paper records when, in addition to the general criteria, the following conditions are met: the signed electronic records must contain information associated with the signing that clearly indicates the name of the signer, the date and time when the electronic record was signed, and the meaning associated with the signature (i.e., review, approval, responsibility, authorship, etc.); the electronic signatures must be linked to their respective electronic records to ensure that the signatures cannot be excised, copied or otherwise transferred by ordinary means so as to falsify an electronic record; and this information must be subject to the same controls as those for electronic records and must be included as part of any human readable form of the electronic record.

Each of these requirements is necessary to identify the individual during the normal course of business and for unambiguously binding an individual to an electronic record.

8.5 Storage media issues

Electronic records may be transferred from one media format to another during the required period of retention. While EPA has allowed for such transfers in the proposed rule, the GLPs require that any such transfer must occur in a fashion that ensures that the entire electronic record is preserved without modification. As noted earlier, the electronic record includes not only the electronic document itself, but also the required information regarding time and date of receipt, etc. Any method of migrating electronic records from one electronic storage medium to another that fails to meet this criterion will not produce records that meet Federal environmental record retention requirements. A CD-ROM version of a record originally stored on electromagnetic tape would not satisfy Federal record keeping requirements unless the method for transferring the record from one medium to the other employed error-checking software to ensure that the data were completely and faithfully transcribed. The Agency is currently seeking comment on whether this criterion is sufficient to ensure that

the integrity and authenticity of the electronic record are maintained throughout its required record retention period.

8.6 Audit trail

One of the key components of the GLP regulations in ensuring that the trustworthiness of data is an effective audit trail, and the same holds true of electronic records and signatures. The FDA regulations require that the audit trail must be computer generated, not a paper record, and that the trail is an internal part of the application one is using. If one is using a laboratory information management system (LIMS), most of the commercial systems will have an audit trail in which changes are logged behind the scenes and appear only when users must enter the reason for a change. The audit trail has some specific requirements. It must be independent of the operator and cover the lifetime of any electronic record from creation through modification and deletion. When an audited change is made, the audit trail must record who made the change; when the change was made, including the date and local time in hours and minutes, the original data without overwriting, the new entry, and the reason for the change.

The audit trail must be retained with, and as long as, the original electronic records. In addition, the audit trail must be in an appropriate form for review or copying by federal investigators.

9 Considerations for electronic submission

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The use of web-centric information technology systems will allow companies to bring their products to market faster, more efficiently, and ultimately more safely. The process of registering a new drug or pesticide with the regulatory authorities is a long and data-intensive process with numerous stakeholders. Pharmaceutical and agrochemical companies are global organizations, and the trials necessary to support product registration produce a vast amount of data. When a pesticide manufacturer petitions a regulatory agency to register a new insecticide, multiple copies of over 100 individual study reports are submitted to the agency to support the petition. Historically, paper copies have been required to be submitted. The practice of submitting and reviewing electronic copies will still be a new and evolving procedure for the next 5–10 years.

The phenomenal growth of the World Wide Web and Internet has revolutionized the delivery of text and image-based information. All signs point to the idea that this will be the definitive technology for the foreseeable future. The rate of change in computer capabilities will pull us all forward. Some of us may not be in the position to drive such changes but merely will be able to follow. One sees acronyms such as CADDY, PDF, HRML, and XML, but what exactly do they mean? How would an electronic submission function? What would it look like? What are the basic pieces, or building blocks, of an electronic submission?

The success of electronic submissions depends on the ability of industry to publish documents electronically and on paper. These versions must be identical; the electronic version must offer the ease of use of the Web. The current way of thinking is in the direction of ‘how do we get our data on to paper?’. Perhaps a better way to think

is 'how do we get our data into an electronic document that will have the format and appearance of paper but with a structured content?'. Accomplishing this will call for a significant change in the work habits of those who write reports.

When many of us began our careers, reports were prepared for publication using typewriters and a support staff of typists. The first change that introduced electronic capabilities for document production occurred with the introduction of word processing systems such as WANG. While this represented progress, the greatest efficiency gain did not occur until the introduction of the personal computer (PC). With the introduction of the PC into the workplace, we saw the first significant change in how reports were written. Complete control of the publication of a report now can lie solely in the hands of its author. Although some 'Luddites' were reluctant to adopt this new technology, today, a professional preparing a report on a PC is commonplace and almost taken for granted. New skills were required but the gain in efficiency and control over one's document led to a rapid acceptance of the PC and a shift in how businesses organized document production. However, there was no real change in how documents were produced. The thought process is still 'paper first, electronic version second'.

Regulatory agency reviewers have indicated that documents submitted in electronic format should enable the user to view easily a clear and legible copy of the information, enable the user to print each document page by page, as it would have been provided on paper, maintaining fonts, special orientations, table formats, and page numbers, include a well-structured table of contents and allow the user to navigate easily through the submission, and allow the user to search and copy text and images electronically into common word processing documents. To achieve these goals, the pesticide regulatory agencies in North America [EPA, FDA, and Pest Management Regulatory Agency (PMRA)] have required that electronic documents be submitted as a PDF. The PDF's property of capturing the exact intent of a document's design in a 'final form' is critical to providing regulatory agencies with documents that can transform how one currently works with pesticide submissions. PDF enables document submitters to provide things such as annotations, tables of contents, bookmarks, and hyperlinks within a single document and between other documents, links to supplemental files in their original form, and content searching. The ability to cut and paste information for re-use and further analysis is critical. The most important point is the ease of producing PDF documents from any application that has the ability to print, and to have, as a result, an electronic version which is identical to the paper version. Both form and structure are needed in electronic pesticide dossiers, and the report or study is the initial building block. With studies in a PDF format, a dynamic submission dossier can be built containing all of the features needed to reduce effort and gain efficiencies within both the regulatory agencies and the industry.

9.1 Creation of PDF documents

Where does one begin and what are the tools required to publish a study in PDF format? Actually, the process can be relatively simple from the standpoint of a single study. The minimal tools required are Adobe Acrobat, Version 4.0 or later (Adobe Systems), and

a plug-in, such as Impress from Mapsoft Computer Services, to provide document pagination. Acrobat offers several methods for converting an electronic file from virtually any application to PDF. One can use either one of two utilities to make this conversion: PDFWriter or Acrobat Distiller. PDFWriter is a printer driver that converts files directly to PDF from any other software application. Distiller is a tool that converts PostScript files to PDF and provides higher quality output than PDFWriter. PDFWriter is often quicker to use than Distiller. Distiller maintains all the formatting, graphics, and photographic images from the original document, and it provides more precise control over the conversion process than PDFWriter. Thus, Distiller would be the preferred option for complex documents. Creating PDF files using Microsoft Office software is simple, and bookmarks can be created automatically based on standard Word headings. Macros included in the Acrobat installation place a 'Create Adobe PDF' as an option under the 'File/Print' menu of Word and Excel so that one can create PDF documents directly from these applications. The basic steps in the creation of a report in PDF file format are as follows: file conversion from applications containing report components by using PDFWriter or Distiller; assembly of PDF files into final report; scan signature page(s) and insert into final report; final document and table of contents pagination; set additional bookmarks if needed (table of contents) to provide navigation; add document reference numbers [e.g., unique report number or EPA master record identification (MRID) number]; and print the final paper copy from the PDF file.

Just as one can print sections or parts of a report from different applications and assemble the report by inserting the paper pages in their appropriate places, one can also insert pages in PDF format from one PDF into another. This is 'cut and paste' at the page level. PDF can capture the exact intent of a document's design in a 'final form' that is identical with the paper version. This is critical to the success of electronic pesticide submissions. The simplicity of creating a PDF from any application with ease is a very powerful feature. The construction of the different components of a study needs to be considered based on page(s) insertion and deletion capabilities of PDF. Different sections of a report can originate from different applications, e.g., tables from Excel can be combined with text sections from Word by rendering them to separate PDF files and then assembling them together into a single PDF.

PDF is a file format that represents a document in a manner independent of the hardware, operating system, and the application software used to create the document. PDF was developed to allow documents to be transferred and shared across computer platforms. This capability allows one to construct a single document under one common format from many different applications.

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9.2 Benefits of PDF documents

PDF documents can increase the efficiency of a reviewer. Time savings can be obtained for reviewers in preparing data evaluation records owing to the ability to copy and paste (drop and drag) information from the PDF study directly into the review's application.

Acrobat 4.0 has a table/formatted text select tool that allows one to select tables and text in a PDF document and retain the original formatting when the material is

copied (or imported) into other applications. One can specify vertical or horizontal format, the type of text flow, and whether one wants ANSI (simple text) or Rich Text Format (RTF).

Being able to search the dossier for relevant information without having to search manually through several studies page by page is another benefit. Imagine being able to search a chronic toxicology study and track a single animal's reference by being able to see just the pages where the reference occurs. This is a big time saving over reviewing each page of a 2000-page report. The indexing of an entire pesticide submission is possible when using searchable PDF files. This allows full content searching across all documents in a collection. For example, one may search for a metabolite and find where the compound is referenced on all pages in each study of a submission.

For industry, the first and most important benefit is a reduction in time to market or increased efficiency in report production. Over the long term, libraries of studies that permit full content searching (knowledge management) can reduce the time needed to respond to questions. Documents entering an electronic document management system would no longer have to be scanned. The same benefits for the reviewer also apply to industry scientists in being able to move data into a new document by being able to copy and paste formatted data regardless of platform and the application used to create the original study.

9.3 Supplemental files

Supplemental files (review aids) are any data set needed by the reviewer for additional analysis purposes that cannot be readily extracted from the PDF report or additional information in electronic format that would enhance the reviewer's understanding or facilitate presentation of the data.

Examples of supplemental files include the following: data tables from mammalian toxicity studies (e.g., body weights, ante- or postmortem observations, clinical chemistry, tumor incidence); data tables from residue chemistry and environmental fate studies (e.g., analytical method validation, residues reported by sample for crop field trials, dislodgeable foliar residues, residue dissipation, and water monitoring); chemical structures in image and scanner interface specification (ISIS)-compatible format; metabolic pathways (metabolism studies) in ISIS-compatible format; photographs (e.g., slides of crop production or processing, crop injury); full test (including graphics) of analytical methods or study reports; models [spray drift, efficacy, Agricultural Reentry Task Force (ARTF)]; dietary exposure input files (including residue distributions); and Pesticide and Root Zone Model (PRZM) input files.

The assumption was that the summary portion of the report (including text and summary tables) would be saved as a PDF file, directly from the word processor used to create the report, and not scanned from a paper copy with a PDF file created from the image. However, owing to the variety of systems that may be used to create tables and appendices of data in the laboratory, it was recognized that some of these might require that PDF files be created from the scanned paper copy.

The Agency science reviewers feel that the advantages of having supplemental files are many, and that they would increase the efficiency and quality of the scientific

reviews. The reviewers would have less data entry to do to check the reported results and to perform statistical calculations on the data. They could more easily add text and tables and graphics to their reviews. Chemical structures and metabolic pathways would not need to be redrawn to be inserted into reviews or Agency databases. The reviewers could have on-line access to the actual study data at internal EPA meetings. Not needing to retype data would result in increased accuracy in data analyses and in reviews, more efficient use of review time, and a more comprehensive use of the study data.

Some data submitters were concerned about whether the supplemental files would be archived along with the data, and whether the supplemental files would be subject to Freedom of Information Act (FOIA) requests. OPP expects to maintain the CD provided as the electronic data submission, and archive the supplemental files on CD along with the rest of the electronic data submission. However, the official archive format at the present time is paper, and the supplemental files duplicate material in the paper submission. Supplemental files may be released upon FOIA requests after the first registration for the pesticide active ingredient, subject to the requirements of FIFRA 10(g), which include an affirmation statement from the requestor and a notice to the data owner, and exclude any FIFRA CBI.

OPP scientists evaluated PDF submissions and supplemental files. For a pilot electronic data submission, OPP discussed the content of supplemental files with the registrant interested in providing an electronic data submission and worked on the data fields of interest for several toxicology studies. OPP evaluated SAS-XPORT (SAS Transport) files as a neutral file format for data tables. Two programs were evaluated by OPP: DBMA Copy and Stat Transfer, which will transfer the data from the SAS-XPORT format into a format usable by the reviewer.

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OPP scientists are identifying the data elements (or fields) of interest for each study type where the reviewers felt that supplemental files would be useful, are identifying other information for each study type, which would be useful to have as electronic files, and are developing guidance for submission of supplemental files. This guidance is not ready to be published, but the scientists will work with the data submitters to ensure that the supplemental files for the particular study will be useful to the science reviewers. Even when guidance becomes available, OPP intends to be flexible about the format of the supplemental files and will modify the guidance as needed.

OPP has developed a number of guidance documents to inform data submitters how to best prepare electronic submissions of studies. The collection of guidance documents will grow over time and be refined as more experience is gained. The EPA Web site should be consulted for guidance on submission of supplemental files with Electronic Data Submissions at **http://www.epa.gov/pesticides** or **http://www.epa.gov/oppfead1/edsgoals.htm**. The Agency Testing Guidelines have been published at **http://www.epa.gov/OPPTS_Harmonized/**.

In the future, as OPP completes its data systems migration and integration efforts and the Agency resolves data transmission and storage security issues, OPP anticipates additional pilots involving Web-based, forms-based submissions. Full consultation with registrants and OPP program and technical staff will continue to be essential and will guide the pilot selection process.

EPA has progressed in their efforts to establish a legal framework to introduce electronic reporting and record keeping (ERR) for environmental compliance

documents. To this end, EPA has assessed the requirements of all its stakeholders, including industry, state and local governments, environmental groups, and the general public. Addressing the legal issues and establishing the legal framework are the most challenging in moving toward E-Gov for a regulatory agency. EPA's commitment to E-Gov was bolstered by the creation of the new Office of Environmental Information (OEI), which has been operational since late 1999. OEI has been given responsibility for stewardship of EPA's information management, policy, and technology. OEI also leads EPA in promoting and fostering electronic reporting and record keeping for compliance reporting in addition to building the support infrastructure within EPA.

The GPEA of 1998 requires Federal agencies to provide regulated companies the option of reporting or keeping records electronically, including the use of electronic signatures, by October 2003. GPEA is significant for it is the first legal step toward realization of E-Gov and marks the first time electronic signatures have been given legal equivalency with the traditional 'wet-ink-on-paper' signatures. Behind paper-based, wet-ink, signatures, a body of experience has developed over the years to analyze handwritten signatures and to detect forgeries and alterations to documents. As a result of this considerable experience, there is a significant body of case law regarding authentication, data integrity, and nonrepudiation in handling environmental compliance reports with handwritten signatures. For electronic filings, such case law, for the most part, is still being developed. Further, when electronic documents are used as evidence in proceedings, they must first be admissible in a court of law as evidence, and they must also be 'persuasive'. While the GPEA legislation prohibits electronic documents from being excluded as evidence solely because they are electronic, the laws do not ensure that juries will find the evidence persuasive. The Agency has drafted a proposed rule, the Cross-Media Electronic Reporting and Record Keeping Rule (CROMERRR),[7] published in the *Federal Register* in July 2001 for public comment, which is designed, in part, to ensure that electronic documents are trustworthy and reliable and provide the same legal and evidentiary force as their paper counterparts.

The proposed rule's goals are straightforward but challenging. They are to provide the regulated community with the option of submitting electronic reports and maintaining electronic records, including electronic signatures, in lieu of paper reports/records and wet-ink signatures, while ensuring that those electronic reports and records submitted and maintained by the regulated community are reliable and trustworthy and available to EPA and State environmental agencies as required by regulation. The multiple objectives include reducing both costs and burden for regulated companies, allowing flexibility for various approaches and providing freedom to adopt new technologies as they became available.

Generally, the proposed CROMERRR establishes the legal framework. The rule removes the existing regulatory barriers to electronic reporting and electronic record keeping, such as the requirements for 'paper'-based reports, signatures, and records embedded throughout EPA's current regulations. The proposal provides for compliance reports to be submitted and/or records to be maintained electronically, in lieu of paper, so long as the electronic reporting or record keeping satisfies the requirements of the rule. Its approach is to identify performance-based criteria that, to the extent possible, ensure integrity, authenticity, and nonrepudiation of electronic

reports and records, specifying 'technology-neutral' criteria for acceptable electronic reporting and record retention systems.

The rule's scope is intended to cover all EPA environmental compliance programs, both reporting and record keeping requirements. The rule does not stipulate technology, nor does it promulgate any new environmental regulatory requirements. The proposed rule sets general requirements in the form of performance-based criteria for government systems receiving electronically signed reports from regulated entities. The general areas addressed by the proposed performance criteria for government systems include system security, electronic signature method, submitter registration process, electronic signature/certification scenario, transaction record, and system archives.

9.4 Central Data Exchange (CDX)

One of the primary differences between the FDA's and EPA's rules is the creation of the CDX. The EPA's OEI is currently developing the specifications for a CDX that will serve as the Agency's gateway for electronic documents received by the EPA. CROMERRR reports that with respect to the electronic document submission addressed by today's proposal, CDX functions will include the following:

1. Access management – allowing or denying an entity access to CDX.
2. Data interchange – accepting and returning data via various file transfer mechanisms.
3. Signature/certification management – providing devices and mandatory scenarios for individuals to sign and certify what they submit.
4. Submitter and authentication – ensuring that signatures are valid and data are uncorrupted.
5. Transaction logging – providing date, time, and source information for data received to establish chain of custody.
6. Acknowledgement and provision of copy of record – providing the submitter with confirmations of data received.
7. Archiving – placing files received and transmission logs into secure, long-term storage.
8. Error checking – flagging obvious errors in documents and document transactions, including duplicate documents and unauthorized submissions.
9. Translation and forwarding – converting submitted documents into formats that will load to EPA databases, and forwarding them to the appropriate systems.
10. Outreach – providing education and other customer services to the CDX.

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The idea is to provide one way and one place for the regulated community to exchange electronic documents with EPA. The CDX may also provide the platform for State–EPA data exchanges. As with the provisions of the proposed rule, the features and functions of CDX described above will generally be inapplicable to these State–EPA exchanges.

The use of the CDX will require little more than access to a computer with a browser and Internet connection. For organizations that have invested heavily in the computerized management of their environmental data, CDX also is designed to

support substantial automation of the data transfer processes. In addition, the EPA hopes that CDX's centralization of data exchanges will eventually provide the platform for greater integration or consolidation of environmental reporting. To support the various functions of CDX, a number of components will have to be incorporated which include the following: digital signatures based on public key infrastructure (PKI) – PKI is a way of reliably establishing and maintaining the identity of the individual producing digital signatures; a process for registering users and managing their access to CDX – EPA would require entities to register with CDX prior to electronic data submission; a characteristic system architecture – EPA has been guided by three goals in designing the CDX (flexibility in exchanging data, uniformity in signing/certifying submissions, and adequate security for all aspects of CDX operation); electronic data interchange (EDI) standards – transmission of electronic data in a standard syntax, of unambiguous information between computers of organizations; and a characteristic environment in which electronic reporting transactions will be conducted – CDX will allow the submitter to transmit data either through automated file transfer, or via on-screen 'smart forms' provided as a part of the downloaded 'desktop'.

The EPA believes that these building blocks, taken together, do satisfy the criteria that today's proposal specifies for electronic document receiving systems.

9.5 An industry perspective

The efficiency gains from electronic data submission (EDS) in the government review process should translate to shorter application review times for registrants. A government–industry partnership is critical to implementing EDS into the pesticide registration process. The primary purpose for this partnership is to ensure consistency in data submission and review requirements, and compatibility of computer hardware and software. Pilot submissions by registrants must be used to validate specific electronic applications and verify potential efficiency gains.

Awareness of the potential benefits that EDS presents has increased substantially in recent years to where those in the registration process who would be the primary benefactors are becoming more involved. This includes company scientists/registration managers and risk assessors/managers who are showing interest in achieving efficiency gains from EDS in the submission, storage, review, and archiving of pesticide regulatory information in contrast to current paper submissions. The principal goal for implementing EDS is to reduce the time and effort required for registrants to prepare and submit pesticide applications and for the regulatory authorities to review data and reach final registration decisions. This goal is extremely important to registrants because time is money in relation to when a product can be marketed.

The major focus for making EDS a viable tool in the pesticide registration process is to identify those functions where benefits are the greatest for the industry. Improving efficiencies for government reviewers was the consensus. The greatest benefit for registrants lies not with their own functions but in improving efficiencies for government reviewers. Improving the performance of the reviewers results in shorter review times. Efficiency gains in the transmittal and archiving of data submissions are important, but secondary.

The successful implementation of EDS in the registration process demands that regulatory agencies and registrants work together in a cooperative partnership to: identify the primary needs of reviewers and where EDS offers the greatest benefits, find the technology that best supports these needs, ensure that regulatory agencies' and registrants' computer hardware/software, operating procedures, and infrastructure are compatible, and verify efficiency gains and operational compatibility through pilot submission projects.

In the USA, the FDA has valuable experience in EDS applications for pharmaceutical drug registrations that should be evaluated closely for application to pesticide registrations. FDA has a legislative mandate to accept all submissions in electronic format by the end of 2002. The regulations for accomplishing this can be found in the *Federal Register* 21 CFR Part 11. The FDA is very much on its way to meeting this legislative mandate and EPA is looking to see what relevant experience can be utilized for pesticides. The Global Crop Protection Federation (GCPF), a federation of national associations of the crop protection industry, is focusing on greater coordination between North America (Canada and the USA) and the European industry approaches to EDS implementation. This primarily involves defining the needs of the respective regulatory authorities to identify common requirements on which coordinated global industry approaches can be based. GCPF is also considering proposals to the OECD Working Group on Pesticides to begin EDS harmonization projects similar to the ongoing efforts of the North American Free Trade Agreement (NAFTA) Technical Working Group (TWG). The membership of the OECD Working Group on Pesticides consists of pesticide regulatory authorities from 29 countries. Obviously, achieving harmonization within the OECD countries will be much more difficult than achieving harmonization among the three NAFTA countries, but then again, the benefits also will be much greater.

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The fundamental principles behind industry's support for EDS in the pesticide registration process are as follows: there must be recognized benefits to both government reviewers and registrants with applications tailored to those needs where efficiency gains are the greatest; the efficiency gains from EDS must be sufficient to justify initial and recurring investments by registrants for the required computer hardware and software; security protection for confidential business information (CBI) must exist for any government electronic submission system; EDS capabilities should be developed through an industry and government partnership to ensure consistency in data submission and review requirements, and compatibility of computer hardware and software; accuracy and reliability of EDS systems must be ensured; EDS systems must be based on open and flexible standards; pilot submissions should be the basis for verifying that specific EDS applications are operationally viable and provide expected efficiency gains; and the use of EDS by registrants must be optional. At present, neither regulatory agencies nor registrants are in a position to utilize EDS to its full potential. In fact, the full potential of EDS applications really has not been identified or demonstrated to the regulated community. This fact alone defines the need, scope, and objectives of EDS implementation programs. Canada has come close to realizing these benefits, if they have not actually achieved them in total. Consequently, there is a very practical and timely urgency for the US industry to pick up this 'new art'.

9.6 Evaluator needs

The PMRA evaluators determined their needs for conducting an efficient electronic review: navigation – easy-to-use bookmarks and links accessed by point and click; document viewing/printing – high quality, viewable on the screen, and easy to print; document annotation – must be able to add reviewer annotations; data manipulation – ability to manipulate data using spreadsheet or other analytical methods; report generation – ability to reuse information through copy and paste functionality; ergonomics – comfortable screen size, PC, and desktop design; and links to other files – includes links to supplemental files, such as histograms, video, etc.

9.7 United States EDS process

The Office of Pesticide Programs (OPP) of the EPA has embarked on a series of pilots with pesticide registrants to develop a standard and process for accepting and reviewing electronic data submissions. OPP is seeking an electronic submission standard that strikes a good balance between the needs of registrants and data reviewers. The standard must be inexpensive and easy for the wide range of US registrants to implement. It must provide OPP reviewers with easily learned functionality that makes their work more efficient and effective. Other interests to be served include data integrity, protection of confidential business information, and international harmonization. While Adobe Acrobat was selected as the tool for the pilot efforts, keeping pace with emerging technologies has presented additional challenges to the Agency.

During the fall (autumn) of 1999, significant factors converged that gave impetus to OPP's pilot efforts. OPP management needed efficiency improvements to address the high volume and high value of studies and their review and also to meet growing demands on the program for regulatory decisions under statutory deadlines. OPP's technical infrastructure and staff capabilities had matured to the point where technology could be put to more sophisticated use. In October 1998, Congress passed the Government Paperwork Elimination Act (GPEA), which mandates that, by 2003, all Federal government agencies must be prepared to accept electronic submissions of whatever information they require from the public, from the States, or from industry. This must be achieved using open standards and nonproprietary software and hardware to the extent possible. As a result of these factors, OPP initiated a series of pilots to test the use of Adobe Acrobat PDF and related tools as the standard for electronic submission and review of study data. This testing was expected to be a 2-year effort because involving a good cross-section of registrants, studies, and reviewers in the pilots would take time. As of mid-July 2002, 30 electronic submissions have been received by the Agency, the first 20 of which were part of the pilot project. Another 10 or so are expected before the end of 2002.

A number of software packages are designed to save documents as PDF files, and more are likely to emerge in the future. Examples are the Corel WordPerfect version 9 and Adobe products such as PageMaker. At the present time, however, to create a PDF file with the enhanced features desired by OPP, the user needs the software package Adobe Acrobat. This program converts word processing and spreadsheet files as well

as scanned images into PDF and preserves the look and feel of the original. The user can create bookmarks and links as navigation aids and can import electronic versions of tables and spreadsheets that retain their native format.

Adobe Acrobat Reader is software that permits users to view, navigate, search, and print Adobe PDF files on major computer platforms. The software is free and available from Adobe Systems' Web site. Although useful, the Reader does not support all the functionality that OPP's reviewers require. For the pilots, OPP purchased Adobe Acrobat, Version 4.0, to support the review of studies submitted as PDF files. Adobe Acrobat permits review, mark-up, annotation, and extraction of text and tables for editing or other manipulations, in addition to basic viewing, navigation, and printing capabilities.

Adobe Acrobat and PDF emerged as the technology tools for OPP's pilots for a number of reasons. These tools are inexpensive and widely used in areas well beyond the pesticide industry; therefore, they can be considered a de facto standard. Adobe supports their product, seeks user feedback, and continues to increase its functionality. The FDA has selected PDF as a standard for electronic submission of new drug applications. FDA's functions are similar to OPP's, and there is some overlap between the regulated communities of both Agencies. OPP's sister office, the Office of Toxic Substances (OTS), has selected PDF for electronic submission of data. OPP was persuaded by the fact that several major registrants, who submit large data packages, suggested PDF. PDF lends itself to structured formats through the use of a feature called 'bookmarks'. OPP can build on this in the future if more highly structured formats are desired, such as XML. Finally, Adobe PDF is consistent with the basic requirements under GPEA.

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OPP established the following operating principles regarding electronic submission and review of studies: the electronic formatting standard must strike a good balance between registrants' and reviewers' needs, must be inexpensive and easy for registrants to adopt, must provide reviewers with easily learned functions that make their work more efficient and effective, and must build on the lessons learned by others. OPP has benefited from the experiences of the FDA, the OPPT, registrants, PMRA pilot efforts, and early electronic submission efforts in the European Union (EU).

For OPP, the bottom-line question for the program was this: is the electronically assisted review process more efficient and more effective? OPP's first pilot was based on specifications developed by FDA. OPP prepared a document that specified the process by which registrants would organize and name the PDF version of studies on CD and how OPP would in-process them. The in-processing includes virus scans, verifying compliance with formatting requirements, and posting the studies on the OPP local area network (LAN). Registrants needed to certify that the information in the electronic version of the study was the same as the paper version of the study, so a Certification with Respect to Data Integrity was developed. Finally, OPP developed a Reviewer Assessment form that would capture the reviewer's experiences during the pilot. Topics addressed included the learning curve, the performance of the tools, ergonomics, LAN and PC performance, and the efficiency and effectiveness of the tools compared with the 'paper' process.

The results of the first pilot were very encouraging. The reviewer found the software easy to learn in all categories of functionality that were used. Tools supporting navigation, text searches, viewing, and printing were scored 'excellent'. Tools to support

excerpting and editing text were scored 'good'. Tools to support excerpting and editing tables and the manipulation of data for export to Excel were scored 'average'. The reviewer noted that a good knowledge of Excel was needed to obtain the data in a usable form. A 17-in and a 21-in monitor were used at different work stations. It was felt that the use of anything less than a 17-in monitor would be insufficient. OPP LAN system performance and response times were acceptable.

The PDF and Acrobat tools supported the critical requirements of improved effectiveness and efficiency. The reviewer could better analyze the data because checking the data against conclusions and statistics was easier and faster. This was accomplished by switching back and forth between linked tables of raw data and the summaries. Regarding efficiency, the reviewer found improvements in nearly all aspects of the work. Data requiring re-analysis could be dropped into the analysis software via drag and drop rather than by retyping and error checking the data. Selected text from the study could be excerpted and dropped into the review document (with proper attribution) without loss of accuracy that could occur during the usual paraphrasing and summarizing of the information.

The reviewer suggested that PDF tables derived from true tables created by the 'table' function of the original word processing files might be more useful. That suggestion has been added to the technical specifications document. Operating efficiencies will be achieved through the promotion and facilitation of the electronic submission process including the delivery, review, data interchange capability, and archiving of data supporting national pesticide registration. This approach will be implemented using current technology, will consider the needs of reviewers and stakeholders, and will address legal, archival, and other requirements. Sub-groups were formed which are developing and refining guidance on the overall formatting of electronic submissions and typical studies as well as supplemental files. A Web site has been established to make information about OPP's pilot efforts, other workgroup activities, and guidance documents and technical specifications available in one place, at **www.epa.gov/oppfead1/edsgoals.htm#pilots**. As of mid-2002, OPP has set a standard for electronic submission and review of study reports. Meanwhile, pilot efforts currently focus on electronic submission of supplemental data files associated with chronic toxicology studies and label text.

9.8 EPA Office of Enforcement (OE) perspective

In 1990, the FDA published the electronic records and signatures rule as an initiative for the pharmaceutical industry. This allowed the use of electronic signatures in lieu of handwritten ones. This initiative opened the door to fully electronic documents. The rule became effective in August 1997 and addresses both electronic records and electronic signatures.

This regulation including its preamble and the final rule itself detailed the minimal requirements. The interpretation of the rule is stronger than the printed word. The goal of FDA was to require electronic records and e-signatures to be reliable, trustworthy, and available to the Agency for inspection.

Similarly, the EPA is concerned with the quality and integrity of electronic records. EPA's CROMERRR proposal establishes requirements that must be met by the

regulated community who modify, maintain, or transmit electronic records. Procedures and controls would need to be put in place to fulfill the requirements stated in the document. The e-documents will be equivalent to paper documents and admissible as evidence in a court of law.

The EPA's CROMERRR proposal states that electronic records and their corresponding electronic record-retention system must establish the following: the ability to generate and maintain accurate and complete copies of records and documents in a form that does not allow alteration of the record without detection; protection of records without alteration throughout the records retention period; the ability to produce accurate and complete copies of an electronic record and render these copies readily available, in both human readable and electronic form, in the normal course of all business processes in a timely manner, as required by predicate regulations, throughout the entire retention period; the ability to ensure that any record bearing an electronic signature contains the name of the signatory, the date and time of signature, and any information that explains the meaning affixed to the signature; the protection of electronic signatures so that the signature that has been affixed to a record cannot be detached, copied, or otherwise compromised; and use of computer-generated, time-stamped audit trails to record independently the date and time of operator entries and actions that create, modify, or delete electronic records. An audit trail is an important element of any acceptable electronic record. It provides an electronic record of key entries and actions to a record throughout the life cycle of the record. Such audit trail documentation needs to be retained for a period at least as long as that required for the subject electronic records. Audit trail documentation also needs to be available for Agency review. Records are searchable and retrievable for reference and secondary uses, including audits, legal proceedings, third-party disclosures, etc., throughout the entire retention period. Electronic records must be archived in an electronic form which preserves the context, meta data, and audit trail. Depending on the record retention period required in predicate regulations, regulated entities must ensure that complete records, including the related meta data, can be migrated to a new system as needed. Computer systems, including software and hardware, controls, and attendant documentation, must be readily available for Agency inspection, and copies must be maintained for records associated with reports that have been electronically submitted to the Agency's Central Data Exchange or to an Agency-certified electronic report receiving system.

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The e-records rule uses many terms that must be understood to grasp fully the intent of the rule. FDA defines electronic records as 'any combination of text, graphics, data, audio, pictorial, or other information presented in digital form that is created, modified, maintained, archived, retrieved, or distributed by a computer system'. A printout of a computer file is not the actual electronic record itself. Electronic records include other relevant data, such as which user made a change, the time and date that the change was made, etc.

10 Regulatory enforcement of electronic data management

Much has changed over the past 20 years within the world of GLPs, and no one area has seen more change than that of IT. This varying landscape has forced the

Table 2 Possible FIFRA GLP citations that apply to electronic data systems

GLP citation	Interpretation
40 CFR 160.29 (a) Personnel	Personnel not trained to operate electronic data collection system
40 CFR 160.33 Study Director	Problems with GLP compliance for a study always comes back to the Study Director
40 CFR 160. 35 (b) QAU	Unauthorized deviations to the SOPs or protocol
40 CFR 160.61 Equipment Design	Data system is not of appropriate design or adequate capacity to function according to protocol and method requirements
40 CFR 160.63(b)(c) Maintenance and Calibration of Equipment	Lacking SOPs on maintenance, calibration, inspection, or testing of data systems or part of systems
40 CFR 160.81(a) Standard Operating Procedures (SOP) – General	Possible deviations from laboratory SOPs on data systems, lack of SOPs for the use and generation of electronic data capture
40 CFR 160.81(b) (10) Standard Operating Procedures (SOP) – Data Handling	Lacking required SOPs on data handling, storage, and retrieval
40 CFR 160.120(a)(13) – Protocol (Maintaining Records)	Protocol must identify records to be maintained
40 CFR 160.130(e) Conduct of Study	No audit trail for changes to data or audit trail established, i.e., • the actual change was not made • the original entry was obscured • the reason for change was not stated • the individual making the change was not identified No individual identified for direct data input

government to modify existing interpretations of regulations and to develop new regulations such as those proposed in the recent consolidation effort. Among the proposed changes to the new regulations will be sections focusing on laboratory technology. These new subsections will address issues concerning the integrity of data stored and manipulated by computers, data processors, and automated laboratory procedures. In addition, the proposed regulations will focus on current 'state-of-the-art' issues including electronic records and signatures. Until the new regulations become final, GLP investigators will need to apply existing regulations to the electronic records and signatures rule. Table 2 provides some typical citations that an investigator might apply to a facility using an electronic data system in place of a manual recording system.

By publishing the final rule, EPA hopes that CROMERRR will accomplish the following: allow the Agency to comply with the GPEA; provide a uniform, technology-neutral framework for electronic reporting and record keeping; and allow EPA programs to offer electronic reporting and record keeping under EPA and State – EPA programs that do not compromise the enforceability of environmental programs. EPA's final rule will be consistent with FDA's electronic records rule.

As the EPA moves toward the eventual finalization of its own rule, currently scheduled for finalization in the spring of 2003, the pressure will mount for EPA investigators and the regulated community to learn about the benefits of the electronic records and

signatures rule, and also the potential consequence resulting from lack of compliance. Like the FDA, the EPA will see whether a company has identified a plan for bringing their systems and procedures into compliance, whether they have performed an assessment or gap analysis, whether they have the necessary controls in place, and whether they are systematically bringing their systems into compliance. EPA investigators expect to handle any potential rule violation as they handle any other deviation with the current GLP regulations. Each deviation will be assessed individually to determine its nature and extent, its impact on product quality or data integrity, and finally, the company's compliance history. EPA is certain that, with time and patience, the electronic records and signatures rule will become an integral part of doing business that will save time, money, and resources as we continue into the new millennium.

10.1 Harmonization

There has been a significant increase in the amount and complexity of data needed to support the registration of pesticides, which has placed extensive burdens on both regulators and registrants. As a result, there is increasing interest among both national regulatory authorities and registrants for the regulatory authorities to harmonize their registration requirements. To this end, efforts in this area are under way in cooperative government organizations, such as the OECD Working Group on Pesticides, the NAFTA Technical Working Group on Pesticides, and the EU. The stated goal of EPA for international harmonization is to develop common or compatible approaches to the review, registration, and standards-setting processes. EPA states that more harmonized regulatory programs for pesticides will lead to improved food safety, reduced regulatory burden on national governments, strengthened scientific procedures, and fewer trade problems.

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The overall intent of harmonization is that national governments share tasks in the review of a registration petition and mutually accept decisions on study reviews. However, actual approaches to harmonization must be based on a common set of data requirements, common guidelines or protocols for conducting experiments to fulfill each of the data requirements, and common quality control criteria for determining the acceptability of the study reports. Scientific reviews by governments for each study report should be written in a common format that allows for mutual acceptance by all governments involved in the harmonized regulatory process. In addition, the actual implementation of harmonization must be based on the following fundamental principles: ensure the continued use of sound science in the regulation of crop protection products while preserving reasonable protection of intellectual property; provide measurable cost reductions in data generation, e.g., minimization of multiple testing to meet the same data requirement of individual countries; facilitate increased cooperation among regulators; and reduce the time from submission of a registration petition to a final registration decision.

Currently, there are a number of harmonization activities in progress. Most of these involve harmonization of data and dossier formats and tend to be paper based. Harmonization efforts on work sharing are also currently under way among international regulatory authorities. Work sharing is facilitated by common formats for data

submissions and review as well as electronic tools to make the submission assembly and review a more efficient and effective process.

The benefits associated with an international harmonization approach include increased predictability and consistency of review time – a benefit to both industry and government. Also, harmonization of common formats allows evaluators to focus on the science of the reviews where less time is spent looking for information or reformatting information. With the use of electronic tools, evaluators can re-use information through 'copy and paste' techniques.

EDS has an important role in the international harmonization of pesticide registrations in facilitating the shared reviews among countries. Since multiple countries will be involved, this presents an expanded aspect to the issue of EDS systems compatibility.

Coordination is needed on a global scale to fulfill registrants' role and commitment to make EDS a reality in NAFTA and OECD countries. To accomplish all this, well-defined plans need to be developed through an industry–government partnership and followed up with specific actions.

10.2 Canada

Successful implementation of EDS will take a number of years and most likely be an evolving, stepwise process. Government regulatory agencies and registrants have only recognized during the past several years the value of EDS and the importance of working together on its implementation. The Canadian PMRA deserves recognition for advancing this favorable environment and so do the European registrants for their work in developing Computer-Aided Dossier, Delivery, and Supply (CADDY) with their regulatory authorities. What is important now is to build on this recent progress and further identify and commit to plans that 'give direction and make things happen'.

The PMRA began a very aggressive program to implement Electronic Dossier Delivery and Evaluation (EDDE), which is PMRA's term for EDS. PMRA's motivation for the program is driven primarily by a cost-recovery mandate from its legislature. One of the first actions taken by PMRA was to identify the requirements of both its reviewers and registrants. PMRA then made decisions on selecting EDS applications and supporting computer hardware/software technology to meet these requirements. PRMA has been actively seeking pilot submissions to verify its technology and procedural selections.

Another important aspect of the Canadian program is the promotion of systems compatibility, not only with Canadian registrants, but also other national regulatory agencies to facilitate international harmonization of registration processes. PMRA published several guidance documents in May 2001 in support of implementing its EDDE program: Guidance to Applicants for Preparing Electronic Submissions: Part I, An Overview; Part II, Guidance for Industry During Pilot Stage; Part III, Guidance of Evaluator Functional Requirements for Electronic Evaluation; and Part IV, Guidance on the Preparation of Documents for Electronic Exchange. These documents are available on the PMRA Web site at **http://www.hc-sc.gc.ca/pmra-arla**.

Because PMRA and the US regulatory agencies are working closely together on registration harmonization under NAFTA, the US's approach for implementing EDS

is similar to that of PMRA. In addition, the US agenices and PMRA are working together to harmonize EDS applications through their involvement in the NAFTA TWG on Pesticides.

An agrochemical company provided four submission formats to PMRA: the CADDY specification, the PDF format, the PDF viewed in a Web browser, and paper. PMRA reviewers compared these formats in a methodical and unbiased manner. PMRA determined that the PDF format gave evaluators a 23% gain in efficiency over paper. They felt that the results clearly demonstrated that the PDF format provided evaluators with sufficient desktop functionality and improved efficiency.

Although CADDY is a useful tool for the European industry and regulatory authorities, PMRA evaluators did not embrace this tool because they felt that it had limitations as an aid to study review by regulatory staff. The outcome expected from future pilots is the establishment of an electronic submission formatting standard that strikes a good balance in meeting efficiency gains for both registrants and reviewers. The standard must be cost effective and easy for the wide range of North American registrants to implement and must provide reviewers with easily learned functionality that makes their work more efficient and effective.

10.3 European Union

The CADDY system was developed by the EU Member States as a practical, flexible and efficient platform for the electronic submission of regulatory dossiers. The CADDY system is in use within the EU.

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By early 1996, the CADDY group had identified an initial strategic goal for the CADDY system, which was to facilitate, in a cost-effective manner, the use of electronic media. The group formulated the following: the provision of dossiers for plant protection products to regulatory authorities; the long-term archiving of such dossiers; and the accessibility of information contained in such dossiers.

The CADDY group determined that the system should have a flexible transfer interface that served the individual needs and requirements of end users. In addition, the system should be modular, capable of incorporating and integrating new technology, and adaptable to changing regulatory needs. The CADDY specifications were built around the following objectives: that the first release should be very simple and cover only those requirements that were absolutely necessary; the page format (stored dossier pages) and index file format (the indexing system necessary to provide efficient document/information retrieval) should be readable by a wide range of standard applications; the retrieval software should meet the needs of users and allow a CADDY submission to replace a paper copy, if desired; the storage and transfer medium should be CD-ROM, so that a complete dossier could be submitted on 2–3 disks; all the pages of the dossier should be represented as Tag Image File Format (TIFF), which are readable by a wide range of standard imaging applications; and the index information should be represented in a format readable by standard database applications. From the outset, a system that met these objectives was thought to be sufficiently flexible to offer a potential basis for worldwide harmonization of electronic submissions.

Table 3 Components of the CADDY system

Component	Definition
Format specification	Defines the format for compiling a CADDY submission. First version finalized in January 1996. Current version 1.1, September 23, 1997. Freely available
Compilation software	Compiles a CADDY submission. The development of the software necessary to compile a CADDY-compliant submission, in line with the format specification, is the responsibility of industry/commercial organizations
Conformity test software	Reports if a dossier on CD/ROM conforms to the CADDY format specification. Current version Revision 18, April 20, 1998. Freely available
Retrieval software	Allows the submission to be displayed and worked with by Regulatory Agencies and others. First version finalized in February 1997. Current format specification 1.2 (version 1.0), August 1999. Software and support available at nominal cost
Information brochure	Provides an overview of the CADDY project. Current version dated March 1998
Application guide	Describes how to deal with CADDY and what to expect from a CADDY submission. Current version dated April 22, 1998

The retrieval software was developed to allow rapid access to the dossier information and to meet the initial needs of users in both industry and regulatory agencies. The range of functions available to the regulatory evaluator when working with an electronic submission is an important factor in determining its successful adoption. Therefore, the functions implemented in the CADDY retrieval software provide the evaluator with the ability to do the following: display/print dossier details; access to studies via either a hierarchical and expandable table of contents or a study (report) list; bookmark studies; annotate studies with private (evaluator only) or public (all users) comments; conduct bookmark and annotational searches; select and sort studies by using a comprehensive multi-attribute search tool; print study lists and details; print studies and study pages; navigate studies using a 'toolbox' (go to next/previous page, etc.); and select text/figures from studies and use optical character recognition to export selections to work processor/spreadsheet files. The main components of the CADDY system are shown in Table 3.

To date, 53 organizations worldwide (43 in Europe, eight in North America and one each in Japan and India) have purchased licenses for the retrieval software and help desk support, covering a total of 563 licenses. Organizations range from regulatory authorities, research-based manufacturers, and generic manufacturers to consultancy/contract houses. The system also has been demonstrated to a wide range of interested organizations involved in pesticide regulation, including authorities considering biocides in the EU, the Food and Agriculture Organization (FAO) in Rome, the World Health Organization (WHO) in Geneva, regulatory authorities in Central and Eastern Europe, and the OECD's Working Group on Pesticides.

CADDY has been adopted as the standard for electronic dossier submissions for plant protection products within the EU. Since the release of the retrieval software

in 1997, the UK has received more than 15 CADDY submissions, with similar numbers having been received by other Member States. The system has led to improved efficiency in dossier compilation by industry and significant savings in handling and shipping costs. On this basis, companies have been quick to realize the benefits of electronic dossier assembly. Production of electronic submissions in CADDY format from in-house document management systems, such as Documentum, has reduced the time to assemble dossiers and associated costs. One producer estimated that a recent EU CADDY submission, comprising eight paper copies and 32 CADDY dossier copies, represented a saving of over $100, 000 compared with paper-based dossiers alone.

For regulatory authorities in Europe, the adoption of CADDY has provided a means of alleviating the burden of handling and storing paper-based submissions. Initial experience with the CADDY system has involved its use as an aid to dossier-completeness checking and as a tool for rapid access to underlying studies in support of decision making. So far, the use of CADDY-based submissions for evaluation has focused on discrete parts of the regulatory submission, such as physical chemical properties and methods of analysis.

Although the initial feedback from users in regulatory agencies has been positive, CADDY, like other electronic submission methods, is unlikely to replace fully the need for paper submissions in the near term. Important ergonomic issues remain to be resolved before full evaluations of active substance dossiers will be routinely conducted electronically and a true paperless submission process achieved. In particular, a general handicap of electronic submissions is the readability of the text on-screen. The resolution and size of screens remain a limiting factor for the computer-based evaluation process. This is not a CADDY-specific issue; it is applicable to all types of electronic submissions since screen technology is still not comparable to paper in terms of readability or ease of use for prolonged periods.

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In 1999, to address the needs of regulators and industry to utilize CADDY fully as an evaluation tool, the CADDY group revised the strategic goal to include 'the examination and assessment of dossiers by regulatory authorities'. This acknowledged the desire of the regulators and industry to enhance and develop the potential of CADDY as an evaluation tool. As part of this process, the retrieval software was further enhanced with the release of a 32-bit version with improved functionality and network support.

In order to assess the requirements for the further development of the retrieval software industry, members of the CADDY group examined the current use of CADDY in six European regulatory authorities early in 2000. The analysis found that overall there was significant interest in CADDY; however, only evaluators in the larger authorities had actual experience with the software. Even though the current CADDY functionality was judged by the evaluators in the six countries to be sufficient for their immediate needs, further improvements were identified.

The adoption of CADDY within the EU has provided a sound basis for the transfer of regulatory dossiers between industry and regulators. CADDY has been developed from inception to a workable system in 5 years and represents a significant collaborative effort between industry and the regulators drawn from both North America and Europe. This initial achievement has resulted in substantial reductions in compiling, transporting, and handling costs for EU dossiers and has provided a basis for rapid

dossier access. CADDY is now being further developed to build upon and enhance its capabilities for use as an evaluation tool. Further information on CADDY is available on the European Crop Protection Association (ECPA) Web site at **www.ecpa.be**.

In the electronic millennium that we have just entered, the ability to construct a complete electronic study in a final form identical with the paper copy must be provided. We, as part of the regulated community, must be ready for change. New skills must be learned. IT organizations must provide support for the document production process. We must learn to think electronic first and paper last.

There is nothing more difficult to carry out, nor more doubtful of success, nor more dangerous to handle, than to initiate a new order of things. *Machiavelli, The Prince, 1532*.

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References

1. Electronic Records, Electronic Signatures; Final Rule, 21 CFR Part 11, *Federal Register*, Vol. 62, No. 54, US Food and Drug Administration, Rockville, MD (1997). Also available on the World Wide Web: **http://www.fda.gov/ora/compliance_ref/part11/default.htm**.
2. Guidance for Industry, 21 CFR Part 11; Electronic Records; Electronic Signatures; Maintenance of Electronic Records, Draft Guidance, Food and Drug Administration, Rockville, MD (2002).
3. Federal Insecticide, Fungicide, and Rodenticide Act; Good Laboratory Practice Standards, 40 CFR Part 160, *Federal Register*, Vol. 54, No. 158, US Environmental Protection Agency, Washington, DC (1989).
4. Good Automated Laboratory Practices: Principles and Guidance to Regulations for Ensuring Data Integrity in Automated Laboratory Operations with Implementation Guidance, Office of Information Resources Management, EPA Directive 2185, US Environmental Protection Agency, Washington, DC (1995). Also available on the World Wide Web: **http://www.epa.gov/irmpoli8/irm_galp/index.html**.
5. Current Good Manufacturing Practices in Manufacturing, Processing, Packing or Holding of Drugs and for Finished Pharmaceuticals, Final Rule, 21 CFR Parts 210 and 211, *Federal Register*, Vol. 43, p. 45076, US Food and Drug Administration, Rockville, MD (1978).
6. ISO/IEC Guide 14025, General Requirements for the Competence of Testing and Calibration in Laboratories, International Organization of Standardization, Geneva (2000). Also available on the World Wide Web: **http://www.iso.ch/iso/en/iso9000-14000/index.html**.
7. CROMERRR, Establishment of Electronic Reporting; Electronic Records, Proposed Rule, *Federal Register*, Vol. 66, No. 170, pp. 46161–46195, US Environmental Protection Agency, Washington, DC (2001). Also available on the World Wide Web: **http://www.epa.gov/cdx/cromerr_rule.pdf**.

Compound class

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Alkylenebis(dithiocarbamates)

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1 Introduction

Alkylenebis(dithiocarbamates) compounds, known also as ethylenebisdithiocarbamates (EBDCs), are nonsystemic, low-toxicity pesticides with strong and broad fungicide activity on plant diseases. Developed by Rohm and Haas and E. I. du Pont de Nemours, they are used to protect vegetables and fruit crops.

The European Community guidelines[1] specify that the maximum residue limit (MRL) for alkylenebis(dithiocarbamates) in fruits and vegetables is 0.05 mg carbon disulfide (CS_2) kg^{-1}.

Representative products of this group are mancozeb, nabam, maneb, and zineb. Their chemical names, formulas, and physical-chemical properties[2] are summarized below.

Mancozeb	
Chemical formula	$[—SCSNHCH_2CH_2NHCSSMn—]_x(Zn)_y$
Chemical name (IUPAC)	Manganese alkylenebis(dithiocarbamate) (polymeric) complex with zinc salt
CAS No.	8017-01-07
Melting point	Decomposes in the range 190–204 °C
Solubility	Insoluble in water and organic solvents
Nabam	
Chemical formula	$NaSCSNHCH_2CH_2NHCSSNa$
Molecular weight	256.3
Chemical name (IUPAC)	Disodium alkylenebis(dithiocarbamate)
CAS No.	142-59-6
Melting point	Decomposes on heating
Solubility	$<200\ g\ L^{-1}$ in water, insoluble in organic solvents
Maneb	
Chemical formula	$[—SCSNHCH_2CH_2NHCSSMn—]_x$

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Molecular weight	265.3
Chemical name (IUPAC)	Manganese alkylenebis(dithiocarbamate) (polymeric)
CAS No.	12427-38-2
Melting point	Decomposes on heating
Solubility	Insoluble in water and organic solvents
Zineb	
Chemical formula	$[—SCSNHCH_2CH_2NHCSSZn—]_x$
Chemical name (IUPAC)	Zinc alkylenebis(dithiocarbamate) (polymeric)
Molecular weight	275.8
CAS No.	12122-67-7
Solubility	10 mg L^{-1} in water, insoluble in organic solvents

Other compounds of this group include mancooper, metiram, and propineb.

Because of their polymeric forms, alkylenebis(dithiocarbamates) are insoluble in water and most organic solvents. Additionally, they form strong complexes with different metal ions.[3] No extraction and chromatographic procedure has been reported for the parent compound of this chemical class. These compounds decompose readily under acidic conditions, for example by contact with the fruit or plant juice generated during sample preparation.

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2 Method overview

Two primary types of methods have been developed to determine alkylenebis-(dithiocarbamate) residues in different crops. Both methods are based on the decomposition of the alkylenebis(dithiocarbamates) at elevated temperature in hydrochloric acid and stannous chloride to form carbon disulfide, which is analyzed by either spectrophotometry or gas chromatography (GC).

For the spectrophotometric method, the evolved carbon disulfide is reacted with copper acetate and diethylamine to form a yellow copper complex which can be measured at 435 nm.[4–6] The recoveries range between 70 and 90%. Reproducibility of this method was improved by reducing the time and the mode of sample pretreatment.[7]

Since all alkylenebis(dithiocarbamates) decompose to carbon disulfide by acid degradation, the above analytical methods are not selective. The result is the measured total residues of all alkylenebis(dithiocarbamates) related products. However, this method is recommended as standard method S15 for alkylenebis(dithiocarbamates) by the German Research Association.[8]

Changing the carbon disulfide trapping agent from copper acetate and diethylamine solution to methanolic potassium hydroxide to produce a xanthogenate increases the method's reproducibility and sensitivity.[9–12]

For the GC method, the generated carbon disulfide is analysed using a flame photomeric detector in the sulfur mode. The acid decomposition is carried out in a sealed glass container at 80 °C, and an aliquot of the headspace is injected into a gas chromatograph.[13–17]

This GC method has also been modified by adding an organic solvent trapping step (benzene or cyclohexane [18]) in the sealed glass to trap the generated carbon disulfide. Carbon disulfide is determined by analyzing an aliquot of the organic layer by GC using electron capture detection.

High-performance liquid chromatography (HPLC) with a micellar mobile phase or with a selective pre-column or reaction detection system has also been used to determine alkylenebis(dithiocarbamaes).[19,20] Zineb and mancozeb residues in feed were determined by ion-pair HPLC with ultraviolet (UV) detection at 272 nm.[21,22] These compounds were converted to water-soluble sodium salts with ethylenediaminetetraacetic acid (EDTA) and sodium hydroxide. The extracts were ion-pair methylated with tetrabuthylammonium hydrogensulfate (ion-pair reagent) in a chloroform–hexane solvent mixture at pH 6.5–8.5. The use of an electrochemical detector has also been reported.[23]

A method to determine nabam by HPLC after acidic hydrolysis to ethylenediamine and post-column derivatization with *o*-phthalaldehyde–mercaptoethanol has also been reported.[24]

Gel permeation chromatography with UV detection at 285 nm[25] was used for the determination of alkylenebis(dithiocarbamates) containing the original alkylenebis-(dithiocarbamate) moiety on tomatoes and lettuce treated with maneb and zineb.

Ethylenethiourea (ETU) is a toxic decomposition product/metabolite of alkylenebis(dithiocarbamates). This compound could be generated during processing of treated crops at elevated temperature. Different chromatographic methods to determine the residue levels of ETU have been published. After extraction with methanol, clean-up on a Gas-Chrom S/alumina column and derivatization (alkylation) with bromobutane, ETU residues can be determined by GC with a flame photometric detector in the sulfur mode.[26] Alternatively, ETU residues can also be determined by an HPLC method[27] with UV detection at 240 nm or by liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/tandem mass spectrometry (LC/MS/MS) (molecular ion m/z 103).[28]

The analytical methods specified above, based on the evolution of carbon disulfide from the parent alkylenebis(dithiocarbamates) by hot acid decomposition using spectrophotometry or GC, are accepted as routine methods to determine alkylenebis-(dithiocarbamate) residues. The two methods are described in detail below.

3 Sample preparation

Sample preparation is a very critical step in the determination of alkylenebis-(dithiocarbamate) residues in different matrices, because these compounds decompose in the acidic milieu of fruit or vegetable juices, and the volatile carbon disulfide can be lost. The samples must be kept deep frozen before and during the homogenization. The homogenization time in the plant chopper should be as short as possible. The samples should not be pulverized too finely, and the sample aliquot for analysis should be taken immediately after homogenization. Alkylenebis(dithiocarbamate) residues decrease with time from sample homogenization until the introduction of the stannous chloride–hydrochloric acid solution into the reaction vessel. In order to avoid contamination, only latex gloves should be used during the sample preparation.

4 Analytical method for the determination of alkylenebis(dithiocarbamates) in plant commodities by hot acid decomposition and spectrophotometric determination

4.1 Principle of the method

Samples are hydrolyzed with hydrochloric acid and stannous chloride solution at elevated temperature, and the evolved carbon disulfide is drawn with an air steam through two gas washing tubes in series containing lead acetate and sodium hydroxide solutions and an absorption tube containing an ethanolic solution of cupric acetate and diethanolamine. Lead acetate and sodium hydroxide remove hydrogen sulfide and other impurities. In the absorption tube, the carbon disulfide forms two cupric complexes of *N*,*N*-bis(2-hydroxyethyl)dithiocarbamic acid with molecular ratios $Cu : CS_2$ of 1 : 1 and 1 : 2. These complexes are measured simultaneously by spectrophotometry at 453 nm.

4.2 Apparatus

Electronic analytical and top loading balances, Mettler or equivalent
Sample chopper, Hobart, or equivalent
Three-necked (23/29 joints), 1000-mL reaction round-bottom flask (Pyrex), equipped with an air inlet glass tube, a Liebig (40-cm length) reflux condenser (Pyrex), and a 250-mL glass dropping funnel (Pyrex)
Hemispherical, temperature-regulated, heating mantle, 500-W, Horst or equivalent
Gas washing tubes, in glass, 250-mL (Pyrex), equipped with spherical socket joints
Trapping (absorption) tube, in glass, 250-mL (Pyrex), equipped with spherical socket joints
Vacuum pump with regulator
UV/visible spectrophotometer, Beckman or equivalent
Glass cuvettes, 1- and 10-cm
Graduated cylinders, various sizes
Volumetric flasks, various sizes
Volumetric pipets, various sizes

DropBooks

4.3 Reagents

Diethanolamine, Fluka, code 3190, or equivalent
Ethanol, Merck, code 983, or equivalent
Methanol, Merck, code 5033, or equivalent
Water, distilled in glass
Hydrochloric acid, 37%, Merck, code 317, or equivalent
Stannous chloride dihydrate, Merck, code 7815, or equivalent
Cupric acetate monohydrate, Merck, code 2711, or equivalent
Lead acetate trihydrate, Merck, code 7372, or equivalent

Sodium hydroxide, Merck, code 6495, or equivalent
Potassium hydroxide, Merck, code 5033, or equivalent
EDTA tetrasodium salt tetrahydrate, Merck, code 10964, or equivalent

4.4 Solutions

Sodium hydroxide, 10% in water. Dissolve 10 g of NaOH in 100 mL of distilled water
Lead acetate, 30% in water. Dissolve 30 g of acetate in 100 mL of distilled water
Stannous chloride solution. Dissolve 40 g of reagent in 100 mL of concentrated hydrochloric acid
Stannous chloride–hydrochloric acid solution. Mix 20 mL of stannous chloride solution with 20 mL of concentrated hydrochloric acid (37%) and 200 mL of distilled water
Cupric acetate solution. Dissolve 400 mg cupric acetate in 250 mL of ethanol. Dilute 25 mL of this solution to 100 mL with ethanol
Color reagent solution. Mix 400 mL of ethanol with 120 mL of copper acetate solution and 100 mL of diethanolamine in a 1000-mL volumetric flask. After dissolution, adjust the volume to 1000 mL with ethanol

4.5 Standards and standard solutions

Alkylenebis(ditiocarbamate) certified standards: Dr Ehrenstorfer, Augsburg, or Riedel-de Haën, Seelze, Germany
Carbon disulfide, Merck, code 2214, or equivalent
Carbon disulfide stock standard solution, 25 mg CS_2 mL^{-1}. Prepare the standard solution weekly and store it in a refrigerator at 4 °C. Weigh about 1.25 g (1.0 mL) of carbon disulfide (with a pipet) in a 50-mL volumetric flask containing 40 mL of ethanol. Reweigh the flask to obtain the exact weight of the carbon disulfide, and adjust the volume to 50 mL with ethanol. Dilute 5.0 mL of this solution to 50 mL with ethanol to obtain a concentration of 2.5 mg mL^{-1}. Dilute 5.0 mL of the latter solution to 250 mL with ethanol to obtain a concentration of 25 μg mL^{-1}. These solutions should be prepared daily.

4.6 Reflux procedure

The apparatus is shown in Figure 1. Install the three-necked reaction round-bottom flask in the electric heating mantle. In one flask neck install the air inlet glass tube, in the second neck install the dropping funnel, and in the third neck install the Liebig reflux condenser. Add 10 mL of 30% lead acetate solution to the first gas washing tube (to remove nontarget gas impurities) and 10 mL of 10% NaOH to the second tube (to remove the hydrogen sulfide). Add 15 mL of color reagent to the carbon disulfide absorption tube. Connect the three tubes, in series, to the upper part of the Liebig condenser. Connect the vacuum pump to the exit of the carbon disulfide absorption trap, and regulate the air flow at about 150 mL min^{-1}. Run cooling water through

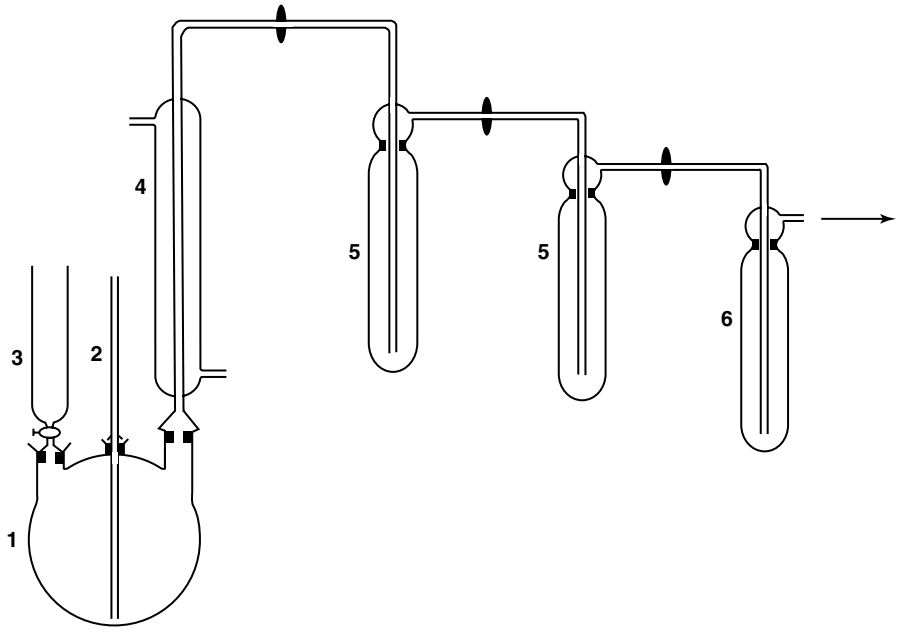


Figure 1 Apparatus for hot acid decomposition of alkylenebis(dithiocarbamates) and distillation of carbon disulfide. 1 = Three-necked round-bottom flask; 2 = air inlet tube; 3 = dropping funnel; 4 = Liebig reflux condenser; 5 = gas washing tubes; 6 = carbon disulfide absorption tube.

DropBooks

the Liebig condenser. Weigh 100–500 g (depending on the residue concentration) of fresh homogenized sample into the reaction three-necked round-bottom flask. Add 150–250 mL (depending on the sample volume) of stannous chloride–hydrochloric acid solution through the dropping funnel, and heat the flask at reflux for 60 min.

4.7 Photometric measurement

After refluxing, disconnect the trapping tube, and transfer the yellow solution into a 25-mL volumetric flask. Rinse the tube with ethanol, and adjust the solution to volume with ethanol. Measure the absorbance of the solution at 435 nm against a blank prepared by diluting 15 mL of color reagent to 25 mL with ethanol. Determine the carbon disulfide content from a calibration curve obtained by plotting carbon disulfide concentrations of different standard solutions on the abscissa versus the absorbance on the ordinate.

4.8 Recovery experiments

As the alkylenebis(dithiocarbamates) are not soluble in water or organic solvents, there are two possible procedures to carry out the recovery experiments. The first is to fortify the solid standard onto the untreated sample in the decomposition vessel, and follow the determination procedure as described above. A second option is to

dissolve the solid standard in aqueous EDTA solution and add a suitable volume of this solution to the untreated sample prior to analysis. For example, prepare a solution of 25 mg of mancozeb in 500 mL of 1% aqueous EDTA. This solution should be stored in a refrigerator at 4 °C. Dilute an aliquot of this solution to the calculated fortification concentration with the aqueous solution of EDTA.

4.9 Limit of quantitation

The limit of quantitation is 0.025 mg CS_2 kg^{-1}, which corresponds to 0.045 mg mancozeb kg^{-1}.

4.10 Methyl xanthate spectrophotometric method

The above method is generally used for large quantities of sample (fruits or vegetables). For smaller volumes of samples and for animal commodities (fat, meat, egg), this method has been slightly modified. The carbon disulfide generated is trapped in a methanolic potassium hydroxide solution instead of ethanolic cupric acetate–diethanolamine color reagent solution. The apparatus and the trapping procedure are the same as specified above, except for the solution of the second gas washing tube, which contains 10 mL of concentrated sulfuric acid. Carbon disulfide is trapped as methyl xanthate (xanthogenate) in a third tube containing 5 mL of 0.5 mol L^{-1} methanolic potassium hydroxide solution. To prepare the potassium hydroxide solution, weigh 14 g of potassium hydroxide into a 500-mL volumetric flask, add 400 mL of methanol, cool the solution to room temperature, and adjust the volume to 500 mL with methanol. An aliquot of the methanolic KOH solution from the absorption tube is transferred into a 25-mL volumetric flask, and the contents are adjusted to volume with methanol.

Measure the intensity of molar extinction at 302 nm against that of a blank solution prepared by diluting 15 mL of methanolic KOH to 25 mL with methanol. Determine the carbon disulfide content from a calibration curve obtained by plotting the carbon disulfide concentrations of different standard solutions on the abscissa versus the absorbance on the ordinate.

The limit of quantification is 0.05 mg CS_2 kg^{-1}.

5 Analytical method for the determination of alkylenebis(dithiocarbamates) in plant commodities by headspace GC and flame photometric (FPD) detection

5.1 Principle of the method

The principle of this determination is the same as for the above spectrophotometric methodology. Alkylenebis(dithiocarbamates) are decomposed with hydrochloric acid and stannous chloride in a closed glass headspace flask at elevated temperature. An

aliquot of the headspace containing the evolved carbon disulfide is injected with a gas-tight syringe directly into the gas chromatograph.

5.2 Apparatus

Electronic analytical and top loading balances, Mettler or equivalent
Sample chopper, Hobart or equivalent
Gas chromatograph for fused-silica capillary or packed columns, equipped with a flame photometric detector (with sulfur filter), Hewlett-Packard, Carlo Erba, or equivalent
Thermostated electric oven, 100 °C, Hereaus or equivalent
Electric heated magnetic stirrer, IKA or equivalent
Magnetic stirrer bar
Graduated cylinders, various sizes
Volumetric flasks, various sizes
Volumetric pipets, various sizes
Electric, temperature-controlled water-bath
Fused-silica capillary columns, 25-m, SE54-CB-1 or OV-1701 (Supelco)
Headspace reaction flasks, in glass, 250-mL, equipped with silicone-rubber septa and screw-caps having a 5-mm hole (Pyrex)
Gas-tight syringes, equipped with valves, 50- to 1000-μL (Supelco).

DropBooks

5.3 Reagents

Ethanol, Merck, code 983, or equivalent
Distilled water
Hydrochloric acid, 37%, Merck, code 317, or equivalent
Stannous chloride dihydrate, Merck, code 7815, or equivalent
EDTA tetrasodium salt tetrahydrate, Merck, code 10964

5.4 Solutions

Hydrochloric acid solution, 5 N. Dilute 430 mL of concentrated hydrochloric acid to 1000 mL with distilled water
Stannous chloride stock solution. Dissolve 40 g of stannous chloride in 100 mL of concentrated hydrochloric acid (37%)
Stannous chloride–hydrochloric acid solution. Mix 20 mL of stannous chloride stock solution with 20 mL of concentrated hydrochloric acid (37%) and 200 mL of distilled water
EDTA solution, 10%. Weigh 100 g of EDTA into a 1-L volumetric flask, and dilute the solution to volume with distilled water

5.5 Standards and standard solutions

Alkylenebis(ditiocarbamate) certified standards: Dr Ehrenstorfer, Augsburg, or Riedel-de Haën, Seelze, Germany

Carbon disulfide, Merck, code 2214, or equivalent

Stock standard solution containing 1 mg mL^{-1} mancozeb. Dissolve about 13.5 mg of mancozeb (taking into consideration the purity of the standard, which is about 74%) in 10 mL of 10% EDTA solution. Prepare this stock solution daily. Prepare working standard solutions by diluting appropriate volumes of the stock solution with 10% EDTA in order to obtain concentrations between 2.5 and 50 μg mL^{-1} mancozeb.

5.6 Headspace procedure

Weigh 50 g of sample into the headspace flask with a magnetic stirrer bar. Add immediately 50 mL of 1.5% stannous chloride solution in hydrochloric acid, and close the vessel with the silicone-rubber septum and the screw-cap. Transfer the headspace flasks into the electric oven (or water-bath) heated at 80 °C for 1 h. After 15 min, take the flasks out of the oven and mix the contents with the magnetic stirrer, heated at 50 °C, for 1 min. Return the flasks to the electric oven and repeat the mixing of the samples every 15 min. After 1 h, take the flasks out of the oven, mix the contents of each flask for 1 min, and inject an aliquot of headspace (100–1000 μL) on to the GC column with a gas-tight syringe.

5.7 Gas-chromatographic conditions

Injector temperature	100 °C
Column temperature	60 °C (isothermal)
Detector temperature	250 °C
Carrier gas	Helium, 30 mL min^{-1}
Combustion gases	Hydrogen, 100–150 mL min^{-1}
	Oxygen, 20–25 mL min^{-1}

Under these chromatographic conditions, the CS_2 retention time is about 3 min on a fused-silica capillary column and about 2 min on a Teflon Chromosil 330 packed column.

5.8 Recovery experiments

Add small volumes of working standard solutions to headspace flasks containing untreated samples, and proceed as described above. The concentration of mancozeb in the samples is calculated by linear regression by plotting the logarithm of the chromatographic peak area versus the logarithm of the amount (in nanograms) of CS_2 injected. From the stoichiometry of the reaction, 1 mol of mancozeb gives 16 mol of CS_2. The conversion factor for mancozeb into CS_2 is 0.557 (1 g of mancozeb yields 0.557 g of CS_2).

6 Conclusions

Alkylenebis(dithiocarbamates) are not stable and decompose on contact with acidic fruit and vegetable juice generated during the sample preparation. The sample preparation is a critical step in their determination. The homogenization should be made with frozen samples as rapidly as possible followed immediately by the analysis.

The most suitable routine analytical method for the determination of alkylenebis-(dithiocarbamate) residues in fruits and vegetables is hot acid hydrolysis with stannnous chloride and concentrated hydrochloric acid, followed by determination of the evolved carbon disulfide by spectrophotometry or GC.

References

1. *Off. J. Eur. Commun.,* **36**, L211, 1 (1994).
2. 'The Pesticide Manual,' 11th edition, British Crop Protection Council, Farnham (1997).
3. H. Irth, G.J. de Jong, U.A. Th. Brinkman, and R.W. Frei, *J. Chromatogr.,* **370**, 439 (1986).
4. E. Cullen, *Anal. Chem.,* **36**, 221 (1964).
5. G.E. Keppel, *JOAC,* **52**, 162 (1969).
6. G.E. Keppel, *JOAC,* **54**, 528 (1971).
7. J. Vuik, R. van Dinter, and R.H. de Vos, *J. Agric. Food. Chem.,* **40**, 604 (1992).
8. Deutsche Forschungsgemeinschaft, 'Rückstandsanalytik und Pflanzenschutzmitteln, Sammelmethoden,' 11 Lieferung, Deutsche Forschungsgemeinschaft, Bonn (1991).
9. W. Schwack and S. Nyanzi, *Z. Lebensm. Unters. Forsch.,* **198**, 3 (1994).
10. W. Schwack and S. Nyanzi, *Z. Lebensm. Unters. Forsch.,* **198**, 7 (1994).
11. W. Schwack and S. Nyanzi, *Z. Lebensm. Unters. Forsch.,* **201**, 69 (1995).
12. Coresta Method, No. 1, *Beitr. Tabakforsch.,* **8**, 107 (1975).
13. H.A. McLeod and K.A. McCully, *JOAC,* **52**, 1226 (1969).
14. T.K. McGhie and P.T. Holland, *Analyst,* **112**, 782 (1987).
15. Committee for Analytical Methods for Residue of Pesticides and Veterinarian Products in Foodstuffs by Headspace Method, of MAFF, Report by the Panel of the Determination of Dithiocarbamate Residues, *Analyst,* **106**, 781 (1981).
16. K. Friedrichs, H.D. Winkeler, and P. Gerhards, *Z. Lebensm. Unters. Forsch.,* **201**, 69 (1995).
17. General Inspectorate for Health Protection, 'Multi-residue Method 5, Part 1,' 1, General Inspectorate for Health Protection, The Hague (1996).
18. R. Pelegri, M. Gamon, R. Coscolla, V. Beltran, and P. Cunat, *Rev. Agroquim. Tecnol. Aliment.,* **31**, 573 (1991).
19. G.F. Kirkbright and F.G.P. Mullins, *Analyst,* **109**, 493 (1984).
20. H. Irth, G.J. de Jong, R.W. Frei, and U.A.Th. Brinkman, *In. J. Environ. Anal. Chem.,* **39**, 129 (1990).
21. K.H. Gustafsson and R.A. Thompson, *J. Agric. Food Chem.,* **29**, 729 (1981).
22. Y. Kunugi, *Tokyo Hishiryo Kensasho,* **19**, 56 (1994).
23. H. van Lishaut and W. Schwack, in 'Abstracts of IUPAC 9th International Congress,' London, 7B-005 (1998).
24. C. Miles and M. Zhou, *JOAC,* **74**, 384 (1991).
25. J. Pflugmacher and W. Ebing, *Z. Lebensm. Unters. Forsch.,* **170**, 349 (1980).
26. J.H. Onley, *JOAC,* **60**, 1111 (1977).
27. H. Diserens and M. Henzelin, Poster presented at the 1st European Pesticide Residue Workshop, Alkmaar, The Netherlands (1996).
28. S.J. Hird, S. Brewin, and A.R.C. Hill, in 'Abstracts of IUPAC 9th International Congress,' London, 7B-006 (1998).

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Multi-residue methods (S19) to measure azole fungicides in crop samples

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1 Introduction

The importance of the azoles has increased during the last few years. In particular, they are used as systemic pesticides in the control of mildew in cereal crops. In general, azole compounds exhibit good activities as leaf fungicides, for seed treatment and for the post-harvest fruit treatment. Triazole derivatives represent one of the most important categories of fungicides to date, owing to their excellent protective, curative and eradicant activities against a wide range of crop diseases. The common structural features responsible for this fungicide class include an imidazole or a 1,2,4-triazole ring.[1]

The analysis of azole compounds is becoming increasingly important. For the regulation of their residues, the multi-method Deutsche Forschungsgemeinschaft (DFG) S19 is used throughout Europe. Within the last few years, this method has been validated for many new azole fungicides in various crop matrices. The multi-residue method and the most important procedural details for the detection and determination of azole compounds are described below. Some important properties are shown in Table 1.

2 Introduction to the method

Multi-residue Method S19 of the DFG Manual,[2,3] including Cleanup Procedure XII-6 (gel-chromatographic cleanup), [4] has been used successfully in many laboratories because of its broad applicability for the gas-chromatographic determination of pesticide residues in foodstuffs. DFG method S19 is also included in the respective European Standards.[5,6] Subsequently, a modification of the extraction and partition step has been implemented.[7] The modified method requires less experimental effort and eliminates the use of dichloromethane, which is an undesirable solvent for toxicological and ecological reasons. As the results from validation studies demonstrate,

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Table 1 Chemical information on representative azole and structurally related compounds

Compound	CAS No.	Molecular formula	Log K_{ow}	GC	ECD sensitivity[a]	NPD sensitivity[a]	*m/z* (electron ionization)	Mol. wt
Azaconazole	60207-31-0	$C_{12}H_{11}Cl_2N_3O_2$	2.17 (pH 6.4)	Yes	×	××	281, 222, 221, 219, 217, 175, 173, 145	300.1
Bitertanol A	70585-36-3	$C_{20}H_{23}N_3O_2$	4.04	Yes	×	×	170, 115, 141, 168, 171, 250, 337	337.4
Bitertanol B	70585-38-5	$C_{20}H_{23}N_3O_2$	4.15	Yes	×	×	170, 115, 141, 168, 171, 250, 337	337.4
Bromuconazole	116255-48-2	$C_{13}H_{12}BrCl_2N_3O$	3.24	Yes	×	××	173, 175, 295, 297, 293, 214	377.1
Cyproconazole, *R**, *R**	94361-06-5	$C_{15}H_{18}ClN_3O$	2.91	Yes	××	××	222, 224, 139, 125, 82, 111	291.8
Cyproconazole, *R**, *S**	94361-07-6	$C_{15}H_{18}N_3ClO$	2.91	Yes	××	××	222, 224, 139, 125, 82, 111	291.8
Diclobutrazole	66345-62-8 or 75736-33-3	$C_{15}H_{19}Cl_2N_3O$	3.81	Yes	××	××	270, 272, 207, 161, 159, 301	328.2
Difenoconazole	119446-68-3	$C_{19}H_{17}Cl_2N_3O_3$	4.2 (25 °C)	Yes	××	×	323, 325, 265, 267, 207, 100, 132	406.3
Diniconazole	83657-24-3	$C_{15}H_{17}Cl_2N_3O$		Yes	×	×	268, 270, 234, 232, 159, 165, 123	326.2
Epoxiconazole	106325-08-0	$C_{17}H_{13}ClFN_3O$	3.44 (pH 7)	Yes	××	××	192, 138, 194, 165, 206, 157, 329	329.8
Etaconazole	60207-93-4	$C_{14}H_{15}Cl_2N_3O_2$		Yes	××	××	245, 247, 173, 191, 175, 145, 206	328.2
Fenbuconazole	114369-43-6	$C_{19}H_{17}ClN_4$	3.23	Yes	×	××	129, 198, 125, 211, 282, 102	336.8
Fenbuconazole-*cis*	146887-38-9	$C_{19}H_{16}ClN_3O_2$	3.23	Yes	×	××	129, 198, 282, 125, 102	336.8
Fenbuconazole-*trans*	146887-37-8	$C_{19}H_{16}ClN_3O_2$	3.23	Yes	×	××	129, 198, 282, 125, 102	336.8
Fluotrimazole	31251-03-3	$C_{22}H_{16}F_3N_3$		Yes	×××	××	165, 311, 233, 239, 379, 275, 77	379.4
Fluquinconazole	136426-54-5	$C_{16}H_8Cl_2FN_5O$	3.24 (pH 5.6)	Yes	×××	××	340, 342, 298, 286, 272, 315, 375	376.2
Flusilazole	85509-19-9	$C_{16}H_{15}F_2N_3Si$	3.74 (pH 7)	Yes		×	233, 206, 315, 234, 128, 155, 165	315.0
Flutriafol	76674-21-0	$C_{16}H_{13}F_2N_3O$	2.3 (20 °C)	Yes	×	×	164, 123, 219, 83, 95, 281	301.3
Furconazole	112839-33-5	$C_{15}H_{14}Cl_2F_3N_3O_2$		Yes				396.2
Furconazole-*cis*	112839-32-4	$C_{15}H_{14}Cl_2F_2N_3O_2$		Yes				396.2
Hexaconazole	79983-71-4	$C_{14}H_{17}Cl_2N_3O$	3.9	Yes	××	××	214, 216, 231, 83, 82, 175	314.2
Imibenconazole	86598-92-7	$C_{17}H_{13}Cl_3N_4S$	4.37	Yes	×	×××		411.7
Ipconazole	125225-28-7	$C_{18}H_{24}ClN_3O$	4.21 (25 °C)	Yes				333.9
Metconazole	125116-23-6	$C_{17}H_{22}ClN_3O$	3.25 (25 °C)	Yes	×	×××	125, 70, 83, 55, 138, 153, 179, 319	319.8
Myclobutanil	88671-89-0	$C_{15}H_{17}ClN_4$	2.94 (pH 7–8)	Yes	××	××	179, 152, 219, 245, 288, 206	288.8
Paclobutrazole	76738-62-0	$C_{15}H_{20}ClN_3O$	3.2	Yes	×	××	236, 125, 238, 167, 218, 138, 149	293.8
Penconazole	66246-88-6	$C_{13}H_{15}Cl_2N_3$	3.72 (pH 5.5)	Yes	×××	××	248, 159, 250, 213, 163, 186, 201	284.2
Propiconazole	60207-90-1	$C_{15}H_{17}Cl_2N_3O_2$	3.72 (pH 6.6)	Yes	××	××	259, 173, 261, 191, 193, 69	342.3
Quinconazole	103970-75-8	$C_{16}H_9Cl_2N_5O$		Yes				358.2
Simeconazole	149508-90-7	$C_{14}H_{20}FN_3OSi$		Yes				293.4
Tebuconazole	107534-96-3	$C_{16}H_{22}ClN_3O$	3.7 (20 °C)	Yes	×	×××	250, 125, 252, 163, 307, 207, 307	307.8

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Table 1—*Continued*

Compound	CAS No.	Molecular formula	LogK_{ow}	GC	ECD sensitivity[a]	NPD sensitivity[a]	*m/z* (electron ionization)	Mol. wt
Tetraconazole	112281-77-3	$C_{13}H_{11}Cl_2F_4N_3O$	3.56 (20 °C)	Yes	××	×	336, 338, 207, 171, 159, 137, 101	372.1
Triadimefon	43121-43-3	$C_{14}H_{16}ClN_3O_2$	3.11	Yes	××	×××	208, 57, 85, 128, 181, 293, 154	293.8
Triadimenol	55219-65-3	$C_{14}H_{18}ClN_3O_2$	3.08 (for Isomer A 3.28)	Yes	××	××	168, 112, 128, 238, 70, 208, 150	295.8
Triapenthenol	76608-88-3	$C_{15}H_{25}N_3O$	2.27	Yes		××	206, 70, 124, 91, 137, 109	263.4
Triazbutil	16227-10-4	$C_6H_{11}N_3$		Yes				125.2
Triticonazole	131983-72-7	$C_{17}H_{20}ClN_3O$	3.29 (20 °C)	Yes	××	××	235, 83, 115, 179, 299	317.8
Uniconazole	83657-22-1	$C_{15}H_{18}ClN_3O$	3.67 (25 °C)	Yes	××	××	234, 236, 70, 57, 101, 131, 165	291.8
Uniconazole-P	83657-17-4	$C_{15}H_{18}ClN_3O$		Yes	××	××	234, 236, 70, 57, 101, 131, 165	291.8
Benzimidazoles and thiazoles								
Benomyl	17804-35-2	$C_{14}H_{18}N_4O_3$	1.37	No				290.3
Carbendazim	10605-21-7	$C_9H_9N_3O_2$	1.5	Yes		×	191, 159, 132, 119, 105	191.2
Chlorfenazole	3574-96-7	$C_{13}H_9ClN_2$		Yes				228.7
Cypendazole	28559-00-4	$C_{16}H_{19}N_5O_3$		Yes				329.4
Debacarb	62732-91-6	$C_{14}H_{19}N_3O_4$		Yes				293.3
Fenapanil	61019-78-1	$C_{16}H_{19}N_3$						253.3
Fuberidazole	3878-19-1	$C_{11}H_8N_2O$	2.67 (22 °C)	Yes		×	184, 156, 155, 129, 183, 189	184.2
Mecarbinizide	27386-64-7	$C_{13}H_{16}N_4O_3S$		Yes				308.4
Rabenzazole	40341-04-6	$C_{12}H_{12}N_4$		Yes		×	212, 170, 118, 195, 214	212.1
Thiabendazole	148-79-8	$C_{10}H_7N_3S$	2.39	Yes		××	201, 174, 129, 146, 130, 142	201.3
Ethaboxam	162650-77-3	$C_{14}H_{16}N_4OS_2$		No				320.4
Etridiazole	2593-15-9	$C_5H_5Cl_3N_2OS_1$	3.37	Yes	××	××	211, 183, 177, 248, 220, 140, 250	247.5
Probenazole	27605-76-1	$C_{10}H_9NO_3S$		Yes				223.2
Imidazolines								
Climbazole	38083-17-9	$C_{15}H_{17}ClN_2O_2$		Yes				292.8
Fenamidone	161326-34-7	$C_{17}H_{17}N_3OS$	2.8 (20 °C)	No				311.4
Glyodin	556-22-9	$C_{22}H_{44}N_2O_2$		No				368.7
Imazalil	35554-44-0	$C_{14}H_{14}Cl_2N_2O$	3.82 (pH 9.2)	Yes	×	×	215, 173, 240, 207, 296, 261	297.2
Iprodione	36734-19-7	$C_{13}H_{13}Cl_2N_3O_3$	3.1	Yes	××	×	187, 244, 314, 216, 271, 303	330.2
Pefurazoate	101903-30-4	$C_{18}H_{23}N_3O_4$	3	No				345.4
Prochloraz	67747-09-5	$C_{15}H_{16}Cl_3N_3O_2$	4.12 (nonionized)	Yes	×	×××	180, 308, 310, 266, 268, 70, 198	375.0
Triazoxide	72459-58-6	$C_{10}H_6ClN_5O$	2.04 (23 °C)	Yes		×××		247.7
Triflumizole, *E*, *Z*	68694-11-1	$C_{15}H_{15}ClF_3N_3O$	5.09 (pH 6.5)	Yes	×××	××	287, 218, 278, 73, 206, 179	345.7
Triflumizole, *E*	99387-89-0	$C_{15}H_{15}ClF_3N_3O$	5.09 (pH 6.5)	Yes	×××	××	287, 218, 278, 73, 206, 179	345.7

[a]Detection sensitivity: × × ×, high; ××, good; ×, weak.

this modification has the same broad field of application as the original DFG Method S19 and is included in the Official German Manual as Method L 00.00-34.

In both cases, the entire method consists of four stages: solvent extraction and partition, cleanup by gel permeation chromatography (GPC) and/or mini silica gel column chromatography and gas chromatography (GC) determination. Except for the central GPC, several variations occur at each stage depending on the kind of sample material and the residues to be analyzed. The variations can be combined with each other in a variety of ways according to the requirements.

The extraction and liquid/liquid partition steps are described in Modules E. The extracts are cleaned up by GPC (Module GPC) and additionally, if required, on a small silica gel column (Module C).

The residue-containing eluate from the GPC step is evaporated and analyzed by GC with nitrogen/phosphorus detection (NPD) (Modules D3 and D4) or mass spectrometry (MS) detection. For GC with electron capture detection (ECD) (Module D1), the GPC eluate requires an additional cleanup on a small silica gel column.

2.1 General overview of the various modules

A general overview of the various Modules is given in Tables 2–5.

Table 2 Gel permeation chromatography

Module	Description	Use/application	Examples
GPC	GPC	Extract from E1 to E9	All samples

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Table 3 Cleanup (C)

Module	Description	Use/application	Examples
C1	Column chromatography on a small silica gel column	GPC eluate, PCB[a] residues not expected	
C2	Column chromatography on a small silica gel column	GPC eluate, PCB[a] residues expected	Animal fats

[a]PCB = polychlorinated biphenyl.

Table 4 Detection (D)

Module	Description	Use/application	Examples
D1	GC with ECD	Eluate from C1 or C2	Organochlorine compounds, pyrethroids, PCBs
D2	GC with FPD	GPC eluate or eluate from C1 or C2	Organophosphorus and sulfur-containing compounds
D3	GC with NPD	GPC eluate or eluate from C1 or C2	Organophosphorus and nitrogen-containing compounds
D4	GC with MS	GPC eluate or eluate from C1 or C2	Compounds containing nitrogen, compounds not detectable with D1 to D3

Table 5 Extraction (E)

Module	Description	Use/application	Examples
E1	Extraction and subsequent liquid/liquid partition [3]	Plant material and foodstuffs with a water content exceeding 70 g/100 g and a fat content below 2.5 g/100 g	Fruit, vegetables, juices
E2	Extraction and subsequent liquid/liquid partition [3]	Plant material and foodstuffs with a water content below 70 g/100 g and a fat content below 2.5 g/100 g	Cereals and cereal products, spices, fruit powder
E3	Extraction and subsequent liquid/liquid partition [3]	Plant material and foodstuffs with a water content exceeding 70 g/100 g, a fat content below 2.5 g/100 g and a high acid content	Fruit, tomatoes
E4	Two-stage extraction and liquid/liquid partition [1,2]	Plant material and foodstuffs with a water content exceeding 70 g/100 g and a fat content below 2.5 g/100 g	Fruit, vegetables, juices
E5	Two-stage extraction and liquid/liquid partition [1,2]	Plant material and foodstuffs with a water content below 70 g/100 g and a fat content below 2.5 g/100 g	Cereals and cereal products, spices, fruit powder
E6	Dissolving fat in GPC eluting mixture	Plant and animal fats (containing no water)	Edible fats and oils, essential oils
E7	Extraction in the presence of large amounts of fat [9]	Plant and animal fats with low water content, if the limit of determination is not sufficient with E6, and dry food with a fat content exceeding 2.5 g/100 g	Edible fats and oils, wheat and rye germs, oats, nuts, oilseed
E8	Extraction of fat with n-hexane–acetone [10]	Fat-containing foodstuffs with high water content	Meat, fish, cheese
E9	ASE	Plant material and foodstuffs with a water content below 20 g/100 g and a fat content below 2.5 g/100 g	Tea, cereals and cereal products, spices

ASE = accelerated solvent extraction.

2.2 Identification and confirmation

In multi-residue analysis, an analyte is identified by its relative retention time, e.g., relative to aldrin when using ECD or relative to parathion or chlorpyrifos when using a flame photometric detection (FPD) and NPD. Such relative retention times are taken from corresponding lists for the columns used. Further evidence for the identity of an analyte is provided by the selectivity of the different detectors (Modules D1 to D3), by its elution behavior during column chromatography (Modules C1 and C2) and in some cases even by the peak form in a gas chromatogram. In a specific analysis for only some individual analytes, their retention times are compared directly with the corresponding retention times of the analytes from standard solutions.

As a rule, a confirmatory GC measurement is performed with a capillary column of different polarity and/or a different detector. The mass-selective detector (MS) (Module D4) is especially suitable for the confirmation of results.

Confirmation of the identity of an analyte should be performed particularly in those cases in which it would appear that a maximum residue limit (MRL) has been exceeded or in which a compound seems to be present which is not to expected in the sample being analyzed.

2.3 Calculation

The residue, W_R in mg kg^{-1}, of an identified analyte is calculated using the sample equivalent C_{Ex} from Modules E, the dilution factors F_{GPC} and F_C from Modules GPC and C, respectively, and the concentration C_A from Modules D. The following equation is used:

$$W_R = \frac{C_A}{C_{Ex}} \times F_{GPC} \times F_C \quad (1)$$

where

C_A = concentration of the identified analyte in the sample test solution from Modules D, in µg mL^{-1}
C_{Ex} = sample equivalent in the extract from Modules E, in g mL^{-1}
F_{GPC} = dilution factor (Module GPC)
F_C = dilution factor (Modules C)

3 Extraction

3.1 Module E1: extraction and subsequent liquid/liquid partition for materials with a water content exceeding 70 g/100 g and a fat content below 2.5 g/100 g

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3.1.1 Outline

The sample is extracted with acetone, after addition of water, depending on the natural water content of the material, in order to ensure an acetone to water ratio of 2 : 1 (v/v) during the extraction.

For liquid/liquid partitioning, sodium chloride and a mixture of cyclohexane and ethyl acetate are added to the homogenate. The mixture is again intensively mixed and allowed to stand until the phases separate. An aliquot of the organic phase is dried with sodium sulfate and concentrated. The concentrated residue is mixed with ethyl acetate and the same volume of cyclohexane. Remaining water is eliminated with a mixture of sodium sulfate and sodium chloride, and the solution is filtered. The extract is subjected to cleanup by GPC (Module GPC).

3.1.2 Reagents

Sodium chloride, p.a.
Sodium sulfate, p.a., anhydrous, powder, heated at 550 °C for at least 2 h
Salt mixture: sodium sulfate–sodium chloride (1 : 1, w/w)
Cotton-wool, extracted exhaustively with acetone
Acetone, for residue analysis
Water, doubly distilled or equivalent

Cyclohexane, for residue analysis
Ethyl acetate, for residue analysis
GPC eluting mixture: cyclohexane–ethyl acetate (1 : 1, v/v); if necessary, redistilled as an azeotropic mixture

3.1.3 Apparatus

High-speed homogenizer, e.g., Ultra-Turrax (Janke u. Kunkel, Staufen/Br., Germany)
Glass jar, 500- or 750-mL, with a screw-cap lined with aluminum foil
Graduated cylinder, 250-mL
Round-bottom flask, 500-mL, with ground joint
Glass funnels, 45- and 100-mm diameter
Rotary vacuum evaporator, water-bath temperature 40 °C
Volumetric pipet, 10-mL
Ultrasonic bath
Fluted filter-paper, 6-cm diameter, fast flow rate, extracted exhaustively with acetone
Membrane filter, 0.45-µm pore size, 25-mm diameter (e.g., Chromafil, Type 0-45/25 organic, Macherey-Nagel No. 718 005)

3.1.4 Procedure

With a portion of the sample, determine the water content in grams per 100 g. As an alternative, take the approximate water content from a literature source.

Weigh 25–100 g (m_A) of the sample having a water content of x g/100 g into a glass jar. Add sufficient water to adjust the total water present to 100 g. The amount of water (m_W) to be added is calculated as follows: $m_W = (100 - m_A) \times x/100$. Next, add 200 mL of acetone and homogenize the mixture for 2 min with the homogenizer.

To the homogenate, add 35 g of sodium chloride and exactly 100 mL of the GPC eluting mixture and homogenize the sample again for 1 min. When the phases are clearly separated after 30–60 min, collect the upper organic phase. In case of insufficient phase separation, centrifuge the mixture. Measure out exactly 200 mL (V_{R1}) of the organic phase into a graduated cylinder, and filter this volume through a glass-wool plug layered with ca 100 g of sodium sulfate in a funnel. Collect the filtrate in a 500-ml round-bottom flask, rinse the graduated cylinder and the funnel four times each with ca 20 mL of GPC eluting mixture and add the rinsates to the round-bottom flask. Concentrate the combined filtrate and rinsings using the rotary evaporator to an aqueous residue. To the aqueous residue, add exactly 7.5 mL of ethyl acetate and swirl the flask in order to dissolve any residues adhering to the flask wall (this is facilitated by immersing the flask in an ultrasonic bath). Add ca 5 g of salt mixture to eliminate the remaining water. Swirl the flask and add exactly 7.5 mL of cyclohexane to obtain a total volume of 15.0 mL (V_{end}). Swirl the flask again, allow the salt mixture to settle and filter the solution through a fluted filter-paper or a membrane filter. With the filtrate, proceed as described in Module GPC.

3.1.5 Calculation

The sample equivalent C_{Ex} corresponds to the amount (in grams) of sample material in 1 mL of extract. Calculate C_{Ex} in g mL^{-1} using the following equation:

$$C_{Ex} = \frac{m_A \times V_{R1}}{V_{Ex} \times V_{End}} \quad (2)$$

where

m_A = sample mass, in g
V_{Ex} = volume of the organic phase after extraction and liquid/liquid partition, in mL (as a rule 285 mL, see *Note* below)
V_{R1} = aliquot portion of V_{Ex} taken for further processing, in mL (200 mL)
V_{End} = volume of the final sample test solution, in mL (15 mL)

The residue, W_R in mg kg^{-1}, of an identified analyte is calculated using the sample equivalent C_{Ex} from Modules E, the dilution factors F_{GPC} and F_C from Modules GPC and C, respectively, and the concentration C_A from Modules D. The following equation is used:

$$W_R = \frac{C_A}{C_{Ex}} \times F_{GPC} \times F_C \quad (3)$$

where

C_A = concentration of the identified analyte in the sample test solution from Modules D, in µg mL^{-1}
C_{Ex} = sample equivalent in the extract from Modules E, in g mL^{-1}
F_{GPC} = dilution factor (Module GPC)
F_C = dilution factor (Module C)

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3.1.6 Important points

Samples may be weighed into the glass jars 1 day before the extraction, if the glass jars are tightly closed with a screw-cap and are stored at −20 °C.

If acid-sensitive analytes (e.g., myclobutanil, propiconazole, tebuconazole, fluotrimazole, thiabendazole, carbendazim) are extracted from an acidic material (e.g., citrus fruits, berries, several sorts of apples and tomatoes), only low recoveries are obtained. If the pH of an aqueous homogenate of the material is <5, use Module E3, where the acids are neutralized before the extraction.

For certain analytes (e.g., dichlofluanid and tolylfluanid), the addition of an acid may increase the recoveries. In these cases, set the pH to <2 by mixing the sample with dilute sulfuric acid (w = 10 g/100 mL) before adding the acetone.

The fat content of the material must not exceed 2.5 g/100 g, or the aqueous brine used in the partitioning step will retain small amounts of fat with some residues included, which may result in a loss of analytes.

Note: 285 mL (V_{Ex}) results from 200 mL of acetone and 100 mL of GPC eluting mixture minus 15 mL caused by volume contraction and by loss of acetone in the aqueous phase.

3.2 Module E2: extraction and subsequent liquid/liquid partition for materials with a water content below 70 g/100 g and a fat content below 2.5 g/100 g

3.2.1 Outline

Sufficient water is added to the sample, depending on the natural water content of the material, in order to ensure an acetone to water ratio of 2 : 1 (v/v). The mixture is allowed to stand for ca 30 min and is then extracted with acetone.

For liquid/liquid partitioning, sodium chloride and a mixture of cyclohexane and ethyl acetate are added to the homogenate. The mixture is again intensively mixed and allowed to stand until the phases separate. An aliquot portion of the organic phase is dried with sodium sulfate and concentrated. The concentrated residue is mixed with ethyl acetate and the same volume of cyclohexane. Remaining water is eliminated with a mixture of sodium sulfate and sodium chloride, and the solution is filtered. The extract is subjected to cleanup by GPC (Module GPC).

3.2.2 Procedure

With a portion of the sample, determine the water content in grams per 100 g. As an alternative, take the approximate water content from Table A1 in the Appendix of DFG method S19 [2,3] or from another literature source.

Weigh 10–50 g (m_A) of the sample having a water content of x g/100 g into a glass jar (e.g., 25–50 g for dried fruit and dried vegetables, 10–20 g for spices and tea, 50 g for cereal grains, 25–50 g for skimmed milk powder and 10–15 g for tobacco). Add sufficient water, pre-heated to 40 °C, to adjust the total water present to 100 g. The amount of water (m_W) to be added is calculated as follows: $m_W = (100 - m_A) \times x/100$. Thoroughly stir the mixture in the glass jar with a glass rod and allow the mixture to stand for 30 min. Next, add 200 mL of acetone and homogenize the mixture for 2 min with the homogenizer.

To the homogenate, add 35 g of sodium chloride and exactly 100 mL of GPC eluting mixture and homogenize the sample again for 1 min. When the phases are clearly separated after 30–60 min, collect the upper organic phase. In case of insufficient phase separation, centrifuge the mixture. Measure out exactly 200 mL (V_{R1}) of the organic phase into a graduated cylinder and filter this volume through a glass-wool plug layered with ca 100 g of sodium sulfate in a funnel. Collect the filtrate in a 500-mL round-bottom flask, rinse the graduated cylinder and the funnel four times each with ca 20 mL of GPC eluting mixture and add the rinsates to the round-bottom flask. Concentrate the combined filtrate and rinsings using the rotary evaporator to an aqueous residue. To the aqueous residue, add exactly 7.5 mL of ethyl acetate and swirl the flask in order to dissolve any residues adhering to the flask wall (this is facilitated by immersing the flask in an ultrasonic bath). Add ca 5 g of salt mixture to eliminate the remaining water. Swirl the flask and add exactly 7.5 mL of cyclohexane to obtain a total volume of 15.0 mL (V_{End}). Swirl the flask again, allow the salt mixture to settle and filter the solution through a fluted filter-paper or a membrane filter. With the filtrate, proceed as described in Module GPC. For calculation, see above.

3.2.3 Calculation

The sample equivalent C_{Ex} corresponds to the amount (in grams) of sample material in 1 mL of extract. Calculate C_{Ex} in g mL^{-1} using the following equation:

$$C_{Ex} = \frac{m_A \times V_{R1}}{V_{Ex} \times V_{End}} \quad (4)$$

where

m_A = sample mass, in g
V_{Ex} = volume of the organic phase after extraction and liquid/liquid partition, in mL (as a rule 285 mL, see Note below)
V_{R1} = aliquot portion of V_{Ex} taken for further processing, in mL (200 mL)
V_{End} = volume of the final sample test solution, in mL (15 mL)

Notes: 285 mL (V_{Ex}) results from 200 mL of acetone and 100 mL of GPC eluting mixture minus 15 mL caused by volume contraction and by loss of acetone in the aqueous phase.

The value for the sample equivalent C_{Ex} is required for calculating the content of an identified analyte according to Section 2.2.

3.2.4 Important points

If acid-sensitive analytes (e.g., myclobutanil, propiconazole, tebuconazole, fluotrimazole, thiabendazole, carbendazim) are extracted from an acidic material (e.g., citrus peel, fruit powder), only low recoveries are obtained. If the pH of an aqueous homogenate of the material is <5, use Module E3, in which acids are neutralized before the extraction.

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3.3 Module E3: extraction and subsequent liquid/liquid partition for materials with a water content exceeding 70 g/100 g, a fat content below 2.5 g/100 g and a high acid content (highly recommended for determining acid-sensitive analytes)

3.3.1 Outline

A sample having a pH of <5 is adjusted to pH ≈ 7 by adding sodium hydrogencarbonate. Sufficient water is added to the sample depending on the natural water content of the material in order to ensure an acetone to water ratio of 2 : 1 (v/v). The mixture is then extracted with acetone.

3.3.2 Procedure

With a portion of the sample, determine the water content in grams per 100 g. As an alternative, take the approximate water content from a literature source.

Weigh 25–100 g (m_A) of the sample having a water content of x g/100 g into a glass jar. Adjust the pH to ca 7 (using pH indicator paper) by adding small portions

of sodium hydrogencarbonate. Add sufficient water to adjust the total water present to 100 g. The amount of water (m_W) to be added is calculated as follows: $m_W = (100 - m_A) \times x/100$. Next, add 200 mL of acetone and homogenize the mixture for 2 min with the homogenizer.

To the homogenate, add 35 g of sodium chloride and exactly 100 mL of GPC eluting mixture and homogenize the sample again for 1 min. When the phases are clearly separated after 30–60 min, collect the upper organic phase. In case of insufficient phase separation, centrifuge the mixture. Measure out exactly 200 mL (V_{R1}) of the organic phase into a graduated cylinder and filter this volume through a glass-wool plug layered with ca 100 g of sodium sulfate in a funnel. Collect the filtrate in a 500-mL round-bottom flask, rinse the graduated cylinder and the funnel four times each with ca 20 mL of GPC eluting mixture and add the rinsates to the round-bottom flask. Concentrate the combined filtrate and rinsings using the rotary evaporator to an aqueous residue. To the aqueous residue, add exactly 7.5 mL of ethyl acetate and swirl the flask in order to dissolve any residues adhering to the flask wall (this is facilitated by immersing the flask in an ultrasonic bath). Add ca 5 g of salt mixture to eliminate the remaining water. Swirl the flask and add exactly 7.5 mL of cyclohexane to obtain a total volume of 15.0 mL (V_{End}). Swirl the flask again, allow the salt mixture to settle and filter the solution through a fluted filter-paper or a membrane filter. With the filtrate, proceed as described in Module GPC. For calculation, see above.

3.3.3 Calculation

The sample equivalent C_{Ex} corresponds to the amount (in grams) of sample material in 1 mL of extract. Calculate C_{Ex} in g mL^{-1} using the following equation:

$$C_{Ex} = \frac{m_A \times V_{R1}}{V_{Ex} \times V_{End}} \tag{5}$$

where

m_A = sample mass, in g
V_{Ex} = volume of the organic phase after extraction and liquid/liquid partition, in mL (as a rule 285 mL, see Notes below)
V_{R1} = aliquot portion of V_{Ex} taken for further processing, in mL (200 mL)
V_{End} = volume of the final sample test solution, in mL (15 mL)

Notes: 285 mL (V_{Ex}) results from 200 mL of acetone and 100 mL of GPC eluting mixture minus 15 mL caused by volume contraction and by loss of acetone in the aqueous phase.

The value for the sample equivalent C_{Ex} is required for calculating the content of an identified analyte according to Section 2.2.

3.3.4 Important points

Samples may be weighed into the glass jars 1 day before the extraction, if the glass jars are tightly closed with a screw-cap and stored at −20 °C.

The extraction described above is highly recommended for determining acid-sensitive analytes (e.g., myclobutanil, propiconazole, tebuconazole, fluotrimazole, thiabendazole, carbendazim).

3.4 Module E4: two-stage extraction and liquid/liquid partition for materials with a water content exceeding 70 g/100 g and a fat content below 2.5 g/100 g

3.4.1 Outline

The sample is extracted with acetone, after addition of water, depending on the natural water content of the material, in order to ensure an acetone to water ratio of 2 : 1 (v/v) during the extraction.

3.4.2 Procedure

With a portion of the sample, determine the water content in grams per 100 g. As an alternative, take the approximate water content from a literature source.

Weigh 25–100 g (m_A) of the sample having a water content of x g/100 g into a glass jar. Add sufficient water to adjust the total water present to 100 g. The amount of water (m_W) to be added is calculated as follows: $m_W = (100 - m_A) \times x/100$. Next, add 200 mL of acetone and homogenize the mixture for 2 min with the homogenizer. Add 10 g of Celite to the mixture and homogenize the sample again for 10 s.

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Filter the homogenate with suction through a fast flow-rate filter-paper in a Buchner funnel until more than 200 mL of filtrate is obtained. Apply only gentle suction to avoid the loss of acetone by evaporation; therefore, do not allow the filter cake to pull dry. The filtration should take not more than 1 min.

Measure out exactly 200 mL of the filtrate (V_{R1}) into a graduated cylinder and transfer this volume into a 500-mL separatory funnel. Add 20 g of sodium chloride and shake the funnel vigorously for 3 min. Add 100 mL of dichloromethane, shake the funnel for 2 min and allow the contents to stand for 10 min. (When using a mechanical shaker, add sodium chloride and dichloromethane simultaneously, vent the separatory funnel and shake the mixture for 5 min.) Usually after shaking, some sodium chloride will remain undissolved. Discard the lower aqueous phase. Draw off the organic phase into a 300-mL Erlenmeyer flask, add ca 25 g of sodium sulfate and allow the flask to stand for about 30 min with occasional swirling. Filter the solution through a cotton-wool plug layered with 3 cm of sodium sulfate in a funnel (100-mm diameter) and collect the filtrate in a 500-mL round-bottom flask. Rinse the Erlenmeyer flask and filter twice with 20-mL portions of ethyl acetate, then add the rinsates to the filtrate. Concentrate the combined filtrate and rinsings to 2 mL in a rotary evaporator and evaporate the remaining solvent using a gentle stream of nitrogen. The residue must be free of dichloromethane.

To the dry residue, add exactly 7.5 mL of ethyl acetate and dissolve the residue by gently swirling the flask. Add 2 g of sodium sulfate, swirl the flask again and add exactly 7.5 mL of cyclohexane to obtain a total volume of 15.0 mL (V_{End}). Shake the flask for ca 20 s and filter the solution through a fluted filter-paper or a membrane filter. With the filtrate, proceed as described in Module GPC.

3.4.3 Calculation

The sample equivalent C_{Ex} corresponds to the amount (in grams) of sample material in 1 mL of extract. Calculate C_{Ex} in g mL^{-1} using the following equation:

$$C_{Ex} = \frac{m_A \times V_{R1}}{V_{Ex} \times V_{End}} \quad (6)$$

where

m_A = sample mass, in g
V_{Ex} = total volume of extract, in mL (as a rule 295 mL, see Notes below)
V_{R1} = aliquot portion of V_{Ex} taken for further processing, in mL (200 mL)
V_{End} = volume of the final sample test solution, in mL (15 mL)

Notes: 295 mL for V_{Ex} results from 200 mL of acetone and 100 mL of water minus 5 mL caused by volume contraction.

The value for the sample equivalent C_{Ex} is required for calculating the content of an identified analyte according to Section 2.2.

3.4.4 Important points

If clogging of the filter occurs, collect only a smaller volume of filtrate and take the portion which runs quickly through the filter-paper. Reduce the amounts of sodium chloride and dichloromethane to be added according to the volume collected, and consider the smaller volume in the calculation.

Samples may be weighed into the glass jars 1 day before the extraction if the glass jars are tightly closed with a screw cap and stored at −20 °C.

The fat content of the material must not exceed 2.5 g/100 g or the aqueous brine used in the partitioning step will retain small amounts of fat with some residues included, which may result in a loss of analyte.

3.5 Module E5: two-stage extraction and liquid/liquid partition for materials with a water content below 70 g/100 g and a fat content below 2.5 g/100 g

3.5.1 Outline

Sufficient water is added to the sample, depending on the natural water content of the material, in order to ensure an acetone to water ratio of 2 : 1 (v/v). The mixture is allowed to stand for ca 30 min and is then extracted with acetone.

3.5.2 Procedure

With a portion of the sample, determine the water content in grams per 100 g. As an alternative, take the approximate water content from Table A1 in the Appendix of DFG method S19[2,3] or from another literature source.

Weigh 10–50 g (m_A) of the sample having a water content of x g/100 g into a glass jar (e.g., 25–50 g for dried fruit and dried vegetables, 10–20 g for spices and tea, 50 g for cereal grains, 25–50 g for skimmed milk powder and 10–15 g for tobacco). Add sufficient water, pre-heated to 40 °C, to adjust the total water present to 100 g. The amount of water (m_W) to be added is calculated as follows: $m_W = (100 - m_A) \times x/100$. Thoroughly stir the mixture in the glass jar with a glass rod and allow the mixture to stand for 30 min. Next, add 200 mL of acetone and homogenize the mixture for 2 min with the homogenizer. Add 10 g of Celite to the mixture and homogenize the sample again for 10 s.

Filter the homogenate with suction through a fast-flow filter-paper in a Buchner funnel until more than 200 mL of filtrate is obtained. Apply only gentle suction to avoid the loss of acetone by evaporation; therefore, do not allow the filter cake to pull dry. The filtration should take not more than 1 min.

Measure out exactly 200 mL of the filtrate (V_{R1}) into a graduated cylinder and transfer this volume into a 500-mL separatory funnel. Add 20 g of sodium chloride and shake the funnel vigorously for 3 min. Add 100 mL of dichloromethane, shake the funnel for 2 min and allow the contents to stand for 10 min. (When using a mechanical shaker, add sodium chloride and dichloromethane simultaneously, vent the separatory funnel and shake the mixture for 5 min.) Usually after shaking, some sodium chloride will remain undissolved. Discard the lower aqueous phase. Draw off the organic phase into a 300-mL Erlenmeyer flask, add ca 25 g of sodium sulfate and allow the flask to stand for about 30 min with occasional swirling. Filter the solution through a cotton-wool plug layered with 3 cm of sodium sulfate in a funnel (100-mm diameter) and collect the filtrate in a 500-mL round-bottom flask. Rinse the Erlenmeyer flask and filter twice with 20-mL portions of ethyl acetate and add the rinsates to the filtrate. Concentrate the combined filtrate and rinsings to 2 mL in a rotary evaporator and evaporate the remaining solvent using a gentle stream of nitrogen. The residue must be free of dichloromethane.

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To the dry residue, add exactly 7.5 mL of ethyl acetate and dissolve the residue by gently swirling the flask. Add 2 g of sodium sulfate, swirl the flask again and add exactly 7.5 mL of cyclohexane to obtain a total volume of 15.0 mL (V_{End}). Shake the flask for ca 20 s and filter the solution through a fluted filter-paper or a membrane filter. With the filtrate, proceed as described in Module GPC.

3.5.3 Calculation

The sample equivalent C_{Ex} corresponds to the amount (in grams) of sample material in 1 mL of extract. Calculate C_{Ex} in g mL^{-1} using the following equation:

$$C_{Ex} = \frac{m_A \times V_{R1}}{V_{Ex} \times V_{End}} \tag{7}$$

where

m_A = sample mass, in g
V_{Ex} = total volume of extract, in mL (as a rule 295 mL, see Notes below)
V_{R1} = aliquot portion of V_{Ex} taken for further processing, in mL (200 mL)
V_{End} = volume of the final sample test solution, in mL (15 mL)

Note: 295 mL for V_{Ex} results from 200 mL of acetone and 100 mL of water minus 5 mL caused by volume contraction.

The value for the sample equivalent C_{Ex} is required for calculating the content of an identified analyte according to Section 2.2.

3.5.4 Important points

If clogging of the filter occurs, collect only a smaller volume of filtrate and take the portion which runs quickly through the filter-paper. Reduce the amounts of sodium chloride and dichloromethane to be added according to the volume collected and consider the smaller volume in the calculation.

3.6 Module GPC: gel permeation chromatography

3.6.1 Outline

The extract derived from one of the Modules E is cleaned up by GPC on polystyrene gel Bio-Beads S-X3 using a mixture of cyclohexane and ethyl acetate as the eluent.

3.6.2 Reagents

Bio-Beads S-X3, 200–400 mesh (Bio-Rad Laboratories, Munich, Germany)
Cyclohexane, for residue analysis
Ethyl acetate, for residue analysis
GPC eluting mixture: cyclohexane–ethyl acetate (1 : 1, v/v); if necessary, redistilled as an azeotropic mixture

3.6.3 Apparatus

Automated equipment for GPC, e.g., Gilson/Abimed Clean-Up XL system (Abimed Analysen-Technik, Langenfeld, Germany) or gel chromatograph GPC Autoprep 1002 (Analytical Biochemistry Laboratories, Columbia, MO, USA; supplier Antec, Pinneberg, Germany). In both cases: chromatography column with end adapters, length 40 cm, 25-mm i.d., sample loop 5.0 mL
Glass syringe, 10-mL, with Luer-lock fitting (or disposable polypropylene syringes)
Long-necked round-bottom flask, 150-mL, with ground joint
Pear-shaped flask, 250-mL, with ground joint
Rotary vacuum evaporator, water-bath temperature 40 °C
Pasteur pipets
Graduated test-tubes, e.g., 12–15-mL, with ground stopper

3.6.4 Procedure

Packing gel permeation column. Suspend 50 g of Bio-Beads in the GPC eluting mixture and allow the beads to swell overnight. Pour the suspension all at once into the chromatographic column (capacity ca 180 mL). Once the gel has settled to a height of

ca 32 cm in the column and is free from air bubbles, insert and lower the end adapter down to the bed level. If the gel bed is compressed further after prolonged use, adjust the adapter accordingly. For further details, refer to the manufacturer's instructions.

Cleanup of crude extracts. Inject ca 10 mL of the filtered extract derived from one of the Modules E into the 5-mL sample loop (5.0 mL = V_{GA}).

Elute the gel permeation column with the GPC eluting mixture at 5.0 mL min^{-1}. To do so, set the determined parameters beforehand, e.g.:

Discard ('Dump')	17 min	corresponding to 85 mL
Collect ('Collect')	22 min	corresponding to 110 mL

Discard the 'Dump' phase. Collect the 'Collect' fraction in a 150-mL long-necked round-bottom flask or in a 250-mL pear-shaped flask. Concentrate the fraction to ca 1 mL in a rotary evaporator (rotate slowly, immerse the flask only slightly in the water-bath). Pipet the concentrated solution quantitatively into a ground stoppered graduated test-tube, rinse the flask with ethyl acetate and dilute the solution to 5.0 mL (V_{GE}) with ethyl acetate.

Calculate the dilution factor F_{GPC} using the following equation:

$$F_{GPC} = \frac{V_{GE}}{V_{GA}} \tag{8}$$

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where

V_{GA} = aliquot portion of the extract volume injected on to the GPC column (5 mL)
V_{GE} = final volume of GPC eluate after concentration (e.g., 5 mL)

Note: The dilution factor F_{GPC} is required for calculating the content of an identified analyte according to Section 3.1.5.

3.6.5 Important points

During an analytical cleanup run, the flow-rate must be 5.0 mL min^{-1}. To check the flow-rate, measure the volume of the eluate in a graduated cylinder.

For multi-residue analysis, a wide elution fraction is collected in order to cover as broad a range of analytes as possible. The elution volumes of the analytes to be determined are given in the Table of the Official Method L 00.00-37. For determining only some individual analytes, a smaller 'Collect' volume can be set in order to match the elution volume of the corresponding analyte.

Lipids are eluted in a volume up to ca 100 mL. If no analytes eluting before 100 mL are to be determined, set the 'Dump' volume to at least 100 mL.

With high-fat materials and small 'Dump' volumes, the GPC eluate may contain more than 0.5 g of lipids. This may seriously affect the elution behavior of the analytes when the GPC eluate is cleaned up further with Modules C1 and C2.

3.7 Module C1: column chromatography on a small silica gel column

3.7.1 Outline

The GPC eluate is cleaned up further on a small silica gel column. This cleanup is essential for the GC determination using ECD (Module D1) and may even sometimes be necessary when using NPD (Module D3). The concentrated GPC eluate is transferred on to a small silica gel column and the column is eluted with solvents or solvent mixtures of increasing polarity.

3.7.2 Reagents

Silica gel, deactivated with 1.5% water. Heat silica gel 60, 70–230 mesh (Merck No. 7734), for at least 5 h at 130 °C, allow to cool in a desiccator and store in a tightly sealed container (ground stopper and masking tape) in the desiccator. Weigh 98.5 g of silica gel into a round-bottom flask with ground joint and add 1.5 g of water. Stopper the flask immediately with a ground stopper and shake the flask intensively for 5 min. Connect the flask to a rotary evaporator and, without applying suction, slowly rotate the flask for 2 h. A mechanical shaker can also be used instead of a rotary evaporator. Check the separation efficiency of each silica gel batch according to Section 4.2 of DFG method S19. The silica gel should not be kept longer than 5 days.
Sodium sulfate, p.a., anhydrous, heated at 550 °C for at least 2 h
n-Hexane, for residue analysis
Isooctane, for residue analysis
Toluene, for residue analysis
Eluent 1: n-hexane–toluene (13 : 7, v/v)
Eluent 2: toluene
Eluent 3: toluene–acetone (19 : 1, v/v)
Eluent 4: toluene–acetone (4 : 1, v/v)
Eluent 5: acetone
Eluent 6: acetone (repeat the elution with 6 mL)

3.7.3 Apparatus

Round-bottom flask, 500-mL, with ground joint
Long-necked round-bottom flask, 100-mL, with ground joint
Pear-shaped flask, 25-mL, with ground joint
Chromatographic tube, length 23-cm, 7-mm i.d., with tapered outlet
Graduated test tubes, e.g., 12–15-mL, with ground stopper
Rotary vacuum evaporator, water-bath temperature 40 °C
Volumetric pipet, 10-mL
Pasteur pipet
Ultrasonic bath

3.7.4 Procedure

Preparation of column. Pack the chromatographic tube in the following order: a quartz-wool plug, 1.0 g of silica gel (deactivated with 1.5% water), then a 5–10-mm layer of sodium sulfate. Finally, insert a small amount of quartz-wool on top of the column packing. Before use, rinse the column with 5 mL of n-hexane and discard the eluate.

Cleanup of sample extract. Pipet 2.5 mL of the solution derived from Module GPC into a long-necked round-bottom flask or a pear-shaped flask and add 10 mL of isooctane. By rotating the flask slowly, carefully evaporate the solution to ca 1 mL in a rotary evaporator (water-bath temperature set at 30–40 °C). If an odor of ethyl acetate is still present, add isooctane again and repeat the evaporation. Repeat, if necessary, until no odor of ethyl acetate is present; the ethyl acetate must be completely removed. Allow the solution to drain to the upper surface of the column packing and then place a graduated test-tube under the column.

Using a volumetric pipet, pipet 2.0 mL of Eluent 1 [n-hexane–toluene (13 : 7, v/v)] into the flask. Immerse the flask in an ultrasonic bath and swirl the flask carefully to dissolve the remaining residue adhering to the glass surface. Using the Pasteur pipet, pipet the solution on to the column and retain the flask to be rinsed later. Allow the solution to drain to the upper surface of the column packing and then elute the column with a further 6.0 mL of Eluent 1. After this has eluted, fill the graduated test-tube to 10.0 mL (V_{CE}) with Eluent 1. Stopper the test-tube and shake it. This solution represents the Eluate 1 fraction. Place another graduated test-tube under the column. Using a volumetric pipet, add 2.0 mL of Eluent 2 to the flask. Immerse and swirl the flask in the ultrasonic bath. Using the Pasteur pipet, transfer the solution on to the column and again retain the flask to be rinsed later. Allow the solution to drain to the upper surface of the column packing, and then elute the column with a further 6.0 mL of Eluent 2. After this has eluted, fill the graduated test-tube to 10.0 mL (V_{CE}) with Eluent 2. Stopper the test-tube and shake it. This solution represents eluate 2.

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Continue the elution of the column as above with Eluents 3–5 and make Eluates 3 and 4 up to 10 mL (V_{CE}) with toluene and Eluate 5 up to 10 mL (V_{CE}) with acetone.

Important points. During preparation and storage of Eluents 1, 3 and 4, make sure that the ratio of the solvent components is correct.

The concentrated GPC eluate must not contain any traces of ethyl acetate. Otherwise, the polarity of the eluents for the silica gel column will be too high, resulting in the analytes eluting much earlier (especially in Eluates 1–3). Therefore, addition and evaporation of isooctane may need to be repeated up to three times.

If the concentrated GPC eluate contains large amounts of lipids, this can also shift the analytes into other eluates. To overcome this, the GPC eluate can be distributed over several silica gel columns.

Frequently, only Eluates 1, 2 and 3 are needed for routine multi-residue analyses with ECD (Module D1). The number of eluates to be collected depends on the nature of the analysis in each case.

Eluate 1 can be cleaned up further by shaking the eluate with 1–2 mL of concentrated sulfuric acid in order to determine analytes which are resistant to concentrated sulfuric acid.

If the detectability of an analyte in a final eluate volume of 10 mL is not sufficient for the GC determination, then pipet an aliquot (V_1) of the eluate V_{CE} into a 25-mL pear-shaped flask and concentrate the aliquot to ca 0.5 mL in a rotary evaporator (water-bath temperature 30–40 °C). By rinsing the flask, transfer the concentrated solution quantitatively into a graduated test-tube and dilute the sample to a suitable volume (V_2). Without this concentration step, volumes V_1 and V_2 need not be considered for the equation given below.

If an analyte will not be detectable in a final eluate volume of 10 mL, then elute the respective fraction directly into a 25-mL pear-shaped flask and concentrate the eluate to ca 0.5 mL in a rotary evaporator (water-bath temperature 30–40 °C). By rinsing the flask, transfer the concentrated solution quantitatively into a graduated test-tube and dilute the sample to a suitable volume.

On the silica gel column, fractionation of the analytes according to their polarity occurs together with the cleanup. Thus the presence of an analyte in a particular eluate gives additional information on its identity. In this respect, the silica gel column cleanup of a GPC eluate which has already been analyzed by GC with NPD or MS may be useful. The distribution of the analytes among the Eluates 1–5 is shown in the Table of the Official Method L 00.00-37.

Calculate the dilution factor F_C using the following equation:

$$F_C = \frac{V_{CE} \times V_2}{V_{CA} \times V_1} \tag{9}$$

where

V_{CA} = aliquot portion of the GPC eluate used, in mL (2.5 mL)
V_{CE} = final volume of the cleaned-up extract, in mL (e.g., 10 mL)
V_1 = aliquot portion of the eluate to be concentrated, in mL
V_2 = volume of the concentrated eluate, in mL

Note: The value of the dilution factor F_C is needed for calculating the content of an identified analyte according to Section 3.1.5.

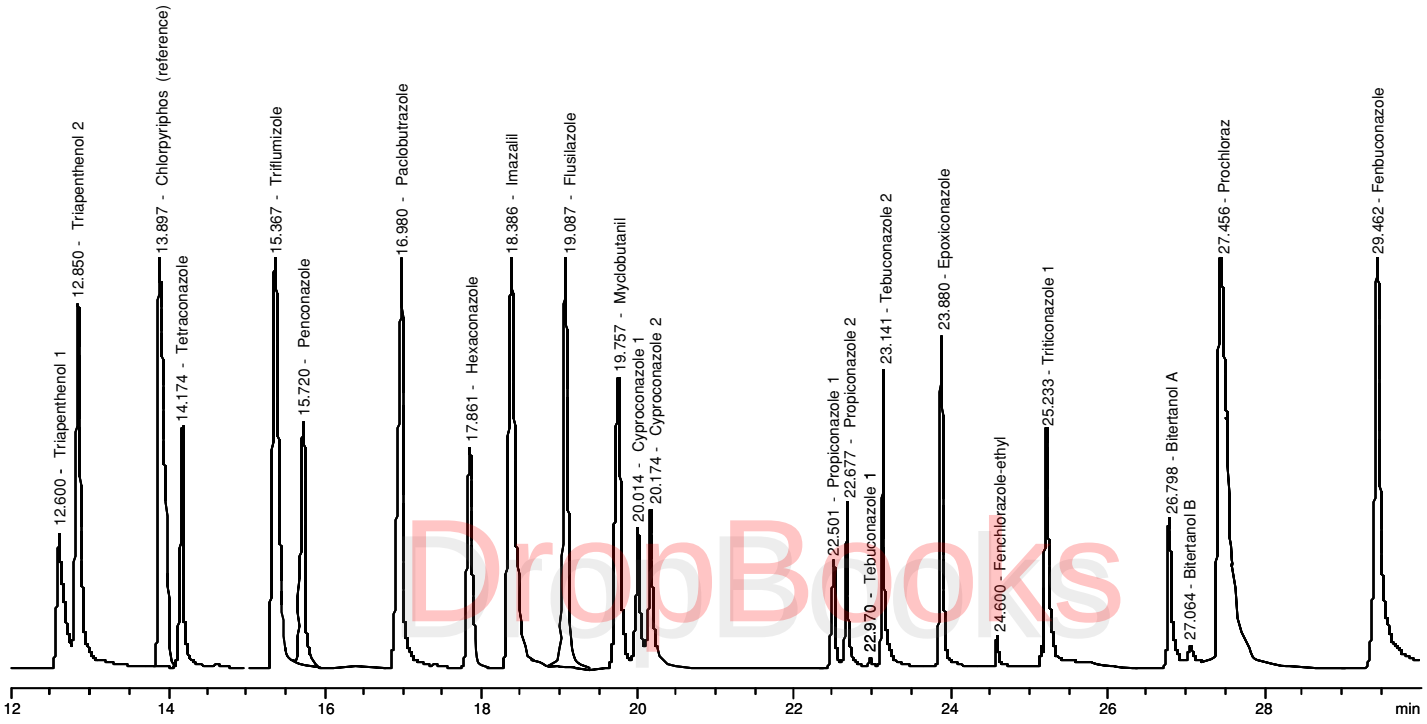
4 Gas chromatography with ECD and NPD

4.1 Procedure

The silica gel column eluates (Module C1 or C2) are injected, if necessary with the addition of an internal standard, into a gas chromatograph followed by ECD or NPD. The determinations can be performed with different gas chromatographs and fused-silica capillary columns.

4.1.1 Apparatus

The following conditions have been proved to be reliable, but they should only be considered as examples. A change in individual parameters does not mean a substantial divergence from the method.



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Figure 1 Azole mixture A analyzed with multi-residue method DFG S19 on an HP 35MS instrument (chlorpyriphos-ethyl as reference)

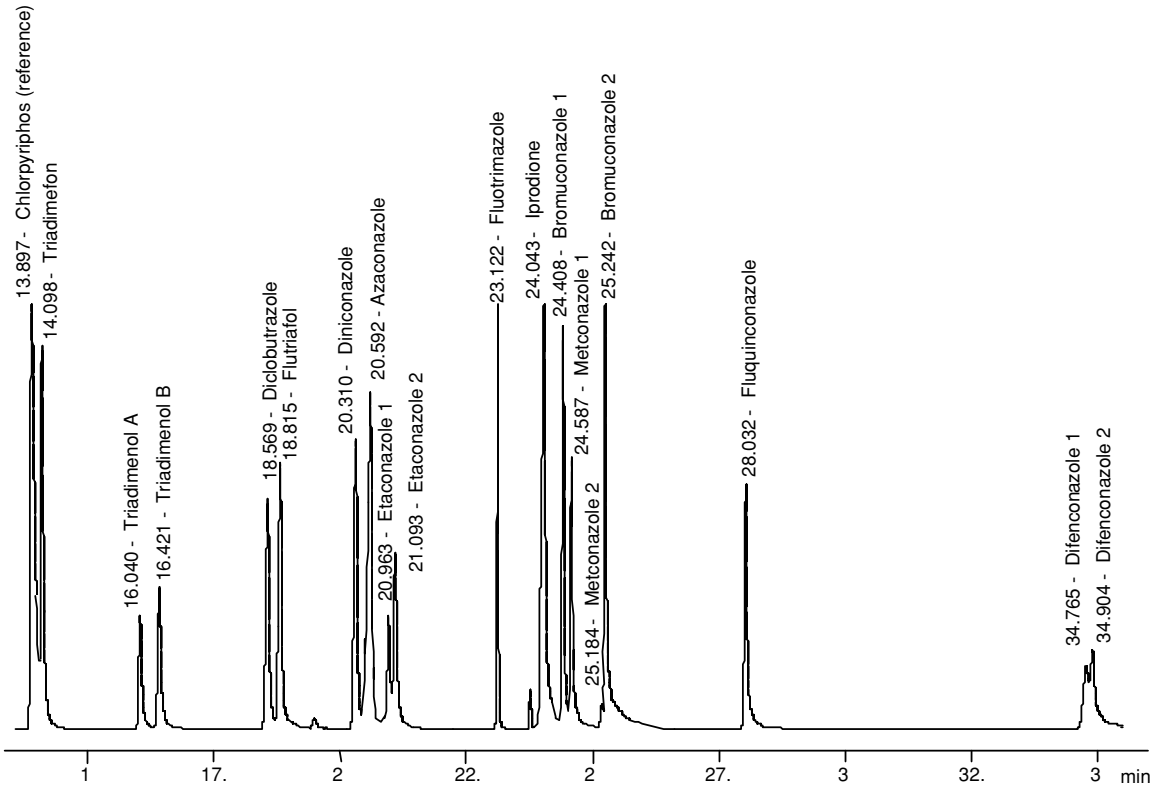


Figure 2 Azole mixture B analyzed with multi-residue method DFG S19 on an HP 35MS instrument (chlorpyriphos-ethyl as reference)

Gas chromatograph 1

Instrument	Hewlett-Packard Model 6890
Column	Fused-silica capillary column, HP 5MS, length 30 m, 0.25-mm i.d., film thickness 0.25-µm (HP No. 190915-433)
Temperature program	70 °C (held for 2 min), increased at 25 °C min^{-1} to 150 °C at 3 °C min^{-1} to 200 °C and at 8 °C min^{-1} to 280 °C (held for 10 min)
Injection, pulsed splitless	50 psi, 0.5 min, purge flow 2.0 min
Injector temperature	240 °C
Detector	NPD, temperature 280 °C
Carrier gas	Helium, 2.9 mL min^{-1}
Purge gas	Nitrogen, 30 mL min^{-1}
Injection volume	2 µL (in pulsed splitless mode)

Gas chromatograph 2

Instrument	Hewlett-Packard Model 6890
Column	Fused-silica capillary column, HP 35MS, length 30 m, 0.32-mm i.d., film thickness 0.25-µm (HP No. 19091G-633)

Table 6 Multi-residue method properties and unusual GC features

Compound	S19 online	GPC (mL)	E1[a]	E2[a]	E3[a]	E4[a]	E5[a]	E6[a]	Comments
Azaconazole	×	120–150				3	2		Fungicide; not stable in ketone solvents
Bitertanol	×	100–130				4	2		Fungicide; 20 : 80 ratio of (1*RS*, 2*RS*) and (1*RS*, 2*SR*) isomers
Bromuconazole	×	105–145				5			Fungicide; mixture of 2 diastereoisomers in proportion 54 : 46
Cyproconazole	×	90–120				1	4		Fungicide; mixture of 2 diastereoisomers in proportion 1 : 1
Diclobutrazole	×	90–120				3	4		Fungicide; 2 diastereoisomers
Difenoconazole	×	70–120				3	2		Fungicide; ratio of *cis*- to *trans*-isomers is in the range 0.7–1.5; partial thermal; decomposition at hot injector
Diniconazole	×	105–125				4	2		Fungicide
Epoxiconazole	×	90–150				3	2		Fungicide; enantiomer pair
Etaconazole	×	90–150				3	2		Fungicide; 2 diastereoisomers
Fenbuconazole	×	105–145				3	3		Fungicide; 2 diastereoisomers; partial thermal decomposition at hot injector; hardly detectable by ECD
Fluotrimazole	×	100–140			4	2			Fungicide; degradation in acidic media; buffered extraction at pH 7 necessary for S19
Fluquinconazole	×				3	2			Fungicide
Flusilazole	×	105–135				4	2		Fungicide; very stable compound; despite containing fluorine atoms, flusilazole was not detectable with ECD
Flutriafol	×	115–135				3	3		Fungicide
Furconazole									Fungicide
Hexaconazole	×	105–135				4	2		Fungicide
Imibenconazole									Fungicide; rapid degradation to 2,4-dichloro-(1*H*-1,2,4-triazol-1-yl) acetanilide
Ipconazole									Fungicide
Metconazole	×	100–125				4	2		Fungicide; mixture of *cis*- and *trans*-isomers (85 : 15)
Myclobutanil	×	110–130				3	3		Fungicide; buffered extraction at pH 7 necessary for S19
Paclobutrazole	×	95–125				3	3		Growth regulator; 2 diastereoisomers
Penconazole	×	110–140				4	2		Fungicide
Propiconazole	×	90–130				4	2		Fungicide; 2 diastereoisomers; buffered extraction at pH 7 necessary for S19; degradation to 1,2,4-triazole
Quinconazole									Fungicide
Simeconazole									Fungicide
Tebuconazole	×	90–120				3	3		Fungicide; buffered extraction at pH 7 necessary for S19
Tetraconazole	×	90–120				4	1		Fungicide
Triadimefon	×	100–130			3	3			Fungicide
Triadimenol	×	100–130				4	2		Fungicide; metabolite from triadimefon
Triapenthenol	×	90–115				5	1		Plant growth regulator
Triazbutil									Fungicide
Triticonazole	×	90–120				2	4		Fungicide
Uniconazole									Plant growth regulator

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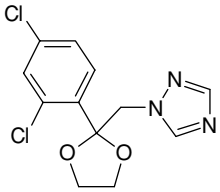
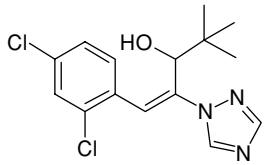
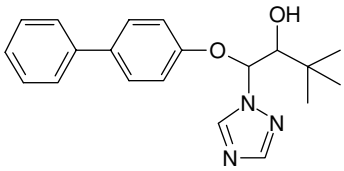
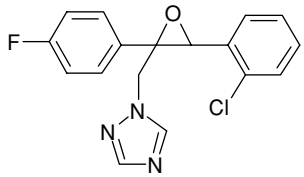
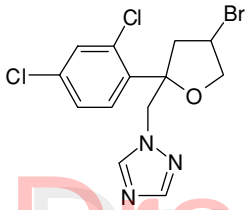
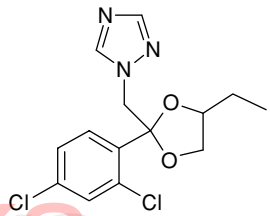
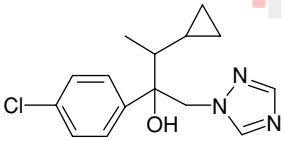
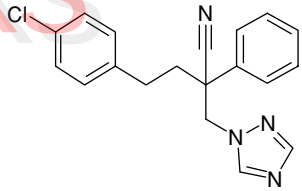
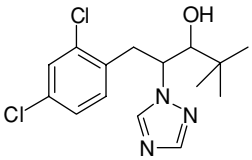
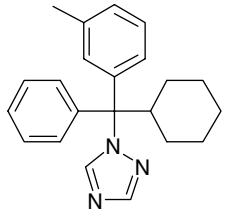
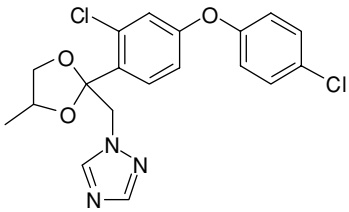
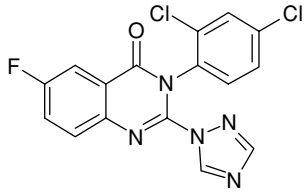
Table 6—*Continued*

Compound	S19 online	GPC (mL)	E1[a]	E2[a]	E3[a]	E4[a]	E5[a]	E6[a]	Comments
Benzimidazoles and thiazoles									
Benomyl	No								Fungicide
Carbendazim	No	135–200							Fungicide; buffered extraction at pH 7 necessary for S19; determinable with HPLC[b]
Chlorfenazole									Fungicide
Cypendazole									Fungicide
Debacarb	No								Fungicide
Fenapanil									Fungicide
Fuberidazole	×	120–160				5	1		Fungicide; very light sensitive; analysis under dark conditions
Mecarbinizide									Fungicide
Rabenzazole	×	120–160			4	1			Fungicide; very light sensitive
Thiabendazole	×	130–160			1	4	2		Fungicide; buffered extraction at pH 7 necessary for S19; determinable with HPLC
Ethaboxam	No								Fungicide
Etridiazole	×	140–160	4	1					Fungicide
Probenazole									Fungicide
Imidazolines									
Climbazole									Fungicide
Fenamidone									Fungicide; diastereoisomers
Glyodin									Fungicide
Imazalil	×	120–150					5	2	Fungicide; very light sensitive; analysis under light exclusion; GC difficult with low concentrations; 20% eluate in E6
Iprodione	×	115–145			5	1			Fungicide; frequently immediately delivers a pre-peak in GC in front of the home signal; after silica gel mini-column treatment the main peak can almost completely disappear
Pefurazoate									Fungicide; slightly unstable to sunlight
Prochloraz	×	120–150				4	2		Fungicide
Triazoxide	×	165–195				5			Fungicide; decomposes in the presence of sunlight
Triflumizole *E*, *Z*	×	80–120			1	4	2		Fungicide; photolytic degradation in aqueous solution; buffered extraction at pH 7 necessary for S19

[a] Recoveries: $5 \approx 90\%$; $4 \approx 60$–90%; $3 \approx 30$–60%; $2 \approx 10$–30%; $1 < 10\%$.
[b] HPLC = high-performance liquid chromatography.

Temperature program	70 °C (held for 1.5 min), increased at 30 °C min^{-1} to 190 °C, at 3 °C min^{-1} to 240 °C and at 30 °C min^{-1} to 280 °C (held for 12 min)
Injection, pulsed splitless	26 psi, 1.5 min, purge flow 2.0 min
Injector program	Programmed temperature vaporization (PTV), 80 °C for 0.2 min, programmed at 700 °C min^{-1} to 220 °C

Table 7 Structures of azole compounds

Name and CAS No.	Structure	Name and CAS No.	Structure
Azaconazole 60207-31-0		Diniconazole 83657-24-3	
Bitertanol 70585-36-3		Epoxiconazole 106325-08-0	
Bromuconazole 116255-48-2		Etaconazole 60207-93-4	
Cyproconazole 94361-06-5		Fenbuconazole 114369-43-6 *cis*: 146887-38-9 *trans*: 146887-37-8	
Diclobutrazole 66345-62-8		Fluotrimazole 31251-03-3	
Difenoconazole 119446-68-3		Fluquinconazole 136426-54-5	

DropBooks

Table 7—*Continued*

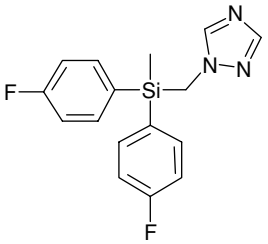
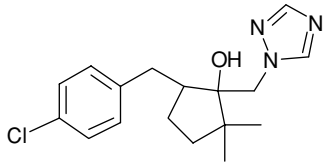
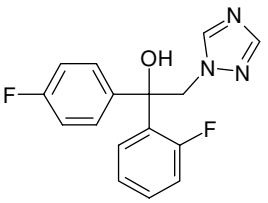
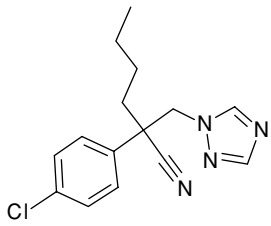
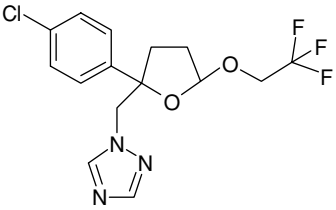
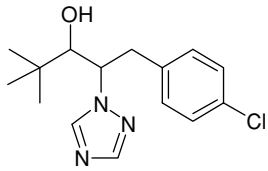
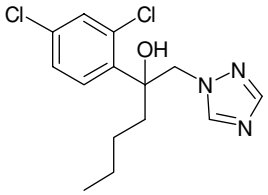
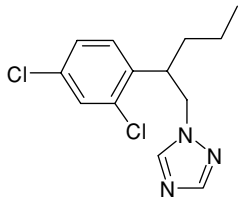
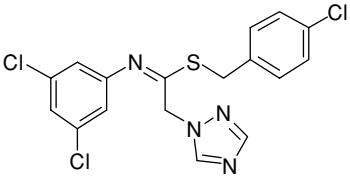
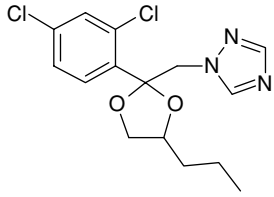
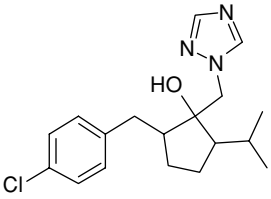
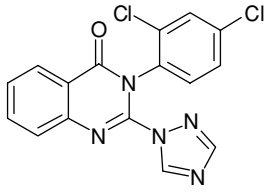
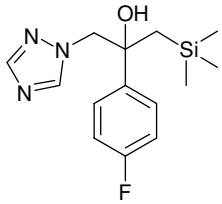
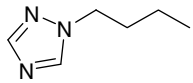
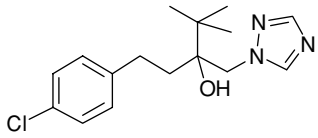
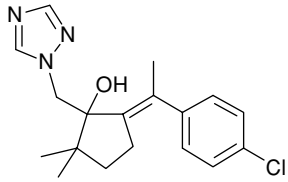
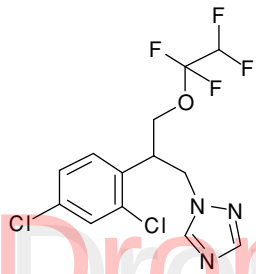
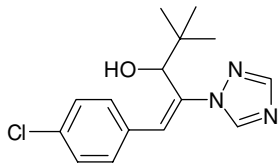
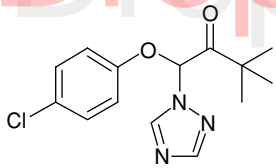
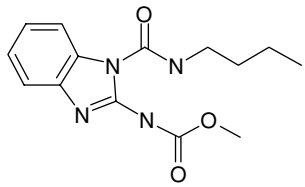
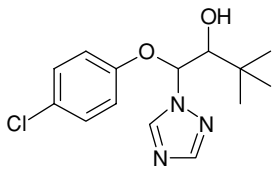
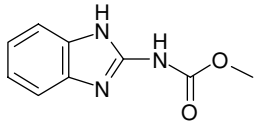
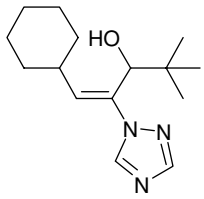
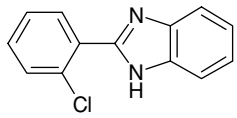
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Flutriafole 76674-21-0		Myclobutanile 88671-89-0	
Furconazole 112839-33-5 *cis*: 112839-32-4		Paclobutrazole 76738-62-0	
Hexaconazole 79983-71-4		Penconazole 66246-88-6	
Imibenconazole 86598-92-7		Propiconazole 60207-90-1	
Ipconazole 125225-28-7		Quinconazole 103970-75-8	

Table 7—*Continued*

Name and CAS No.	Structure	Name and CAS No.	Structure
Simeconazole 149508-90-7		Triazbutil 16227-10-4	
Tebuconazole 107534-96-3		Triticonazole 131983-72-7	
Tetraconazole 112281-77-3		Uniconazole 83657-22-1 Uniconazole-P 83657-17-4	
		Benzimidazoles and thiazoles	
Triadimefone 43121-43-3		Benomyl 17804-35-2	
Triadimenole 55219-65-3		Carbendazim 10605-21-7	
Triapenthenole 76608-88-3		Chlorfenazole 3574-96-7	

DropBooks

Table 7—*Continued*

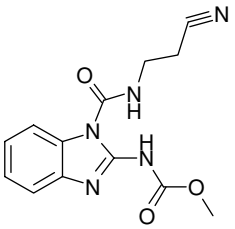
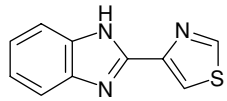
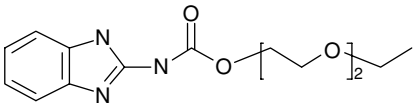
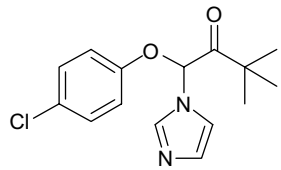
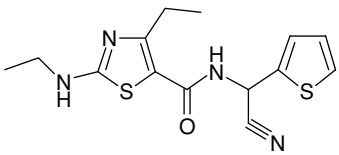
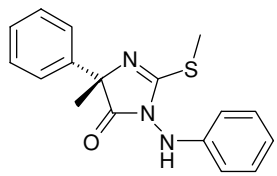
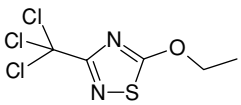
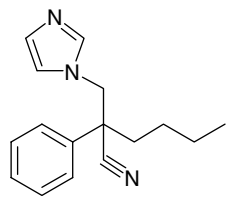
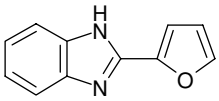
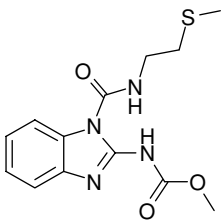
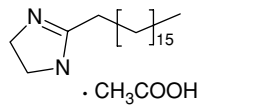
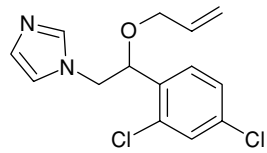
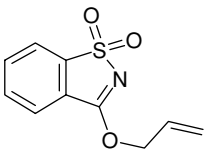
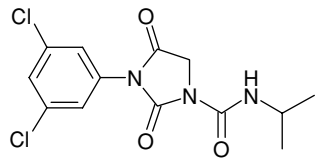
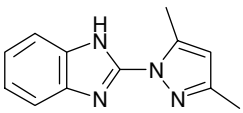
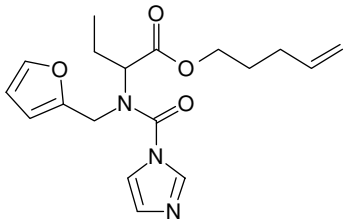
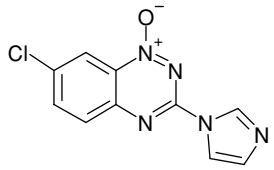
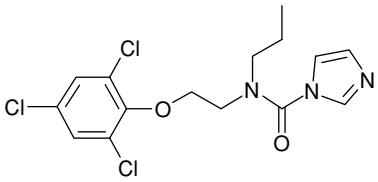
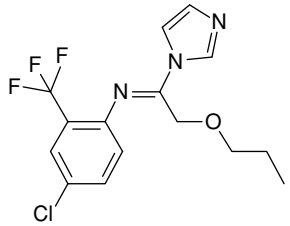
Name and CAS No.	Structure	Name and CAS No.	Structure
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		Imidazolines	
Debacarb 62732-91-6		Climbazole 38083-17-9	
Ethaboxam 162650-77-3		Fenamidone 161326-34-7	
Etridiazole 2593-15-9		Fenapanil 61019-78-1	
Fuberidazole 3878-19-1			
Mecarbinizide 27386-64-7		Glyodin 556-22-9	
		Imazalil 35554-44-0	
Probenazole 27605-76-1			
		Iprodione 36734-19-7	
Rabenzazole 40341-04-6			

Table 7—*Continued*

Name and CAS No.	Structure	Name and CAS No.	Structure
Pefurazoate 101903-30-4		Triazoxid 72459-58-6	
Prochloraz 67747-09-5		Triflumizole, *E*, *Z* 68694-11-1 Triflumizole, *E* 99387-89-0	

	(held for 1.0 min) and at 700 °C min^{-1} to 260 °C (held for 2.0 min)
Detectors 1 and 2	NPD, temperature 280 °C, and ^{63}Ni ECD, temperature 300 °C
Carrier gas	Helium, pressure 14.75 psi, constant flow-rate 1.2 mL min^{-1}
Purge gas	Nitrogen, 30 mL min^{-1}
Injection volume	2 µL (pulsed splitless mode)
Column splitter	After the analytical column; ratio 1 : 5 (ECD : NPD), HP No. 5181-3389

DropBooks

4.1.2 Important points

For this GC determination with ECD, use only a sample test solution which has been adequately subjected to cleanup by silica gel column chromatography or by other means. Eluates 4 and 5 frequently contain considerable amounts of co-extractives, which may affect the evaluation of the chromatograms.

As a rule, the eluate obtained from GPC (Module GPC) can be directly injected for NPD. In special cases, an eluate from the small silica gel column (Module C1) may be recommended to obtain a better quality of chromatograms.

The MS detector is a particularly suitable tool for confirming the identity of an analyte which has been detected with ECD or NPD. Confirmation of identity should be performed particularly in those cases in which the MRL appears to have been exceeded or in which a compound seems to be present which is not expected in the sample being analyzed. In this case, the scan mode is used in order to identify the compound by means of its mass spectrum.

Figures 1 and 2, as examples, show analyses with gas chromatograph 2 and NPD.[8] This detector in combination with column chromatography on a small silica gel column provided a good means to detect the azoles with high selectively and sensitivity.

Important operating parameters of multi-residue method S19 and several important GC features are presented in Table 6.

Table 7 shows the structures of representative azole compounds and several structurally related compounds such as benzimidazoles, thiazoles and imidazolines.

5 Summary

The extraction Module E3 is particularly suitable for the analysis of the partly acid-sensitive azoles (e.g., myclobutanil, propiconazole, tebuconazole, fluotrimazole, thiabendazole, carbendazim). By combining the cleanup step using a GPC column and separation on a small silica gel column, 27 azoles, four benzimidazoles and thiazoles and five imidazolines could be determined with this multi-residue method.

References

1. C. Tomlin (ed.), 'The Pesticide Manual,' twelfth edition, British Crop Protection Council, Farnham, and Royal Society of Chemistry, Cambridge (2000).
2. Deutsche Forschungsgemeinschaft, 'Rückstandsanalytik von Pflanzenschutzmitteln, Stand der 11. Lieferung,' VCH, Weinheim, Sammelmethode S19 (1991).
3. Deutsche Forschungsgemeinschaft, 'Manual of Pesticide Residue Analysis,' VCH Weinheim, Vol. 1, 383–400 (1987); Vol. 2, 317–322 (1991).
4. Deutsche Forschungsgemeinschaft, 'Rückstandsanalytik von Pflanzenschutzmitteln, Stand der 11. Lieferung,' VCH, Weinheim, Aufarbeitungsverfahren XII-6 (1991).
5. DIN EN 1528-3, 'Fettreiche Lebensmittel – Bestimmung von Pestiziden und Polychlorierten Biphenylen (PCB) – Teil 3: Reinigungsverfahren, Verfahren G', Deutsches Institut für Normung, Berlin (1996).
6. DIN EN 12393-2, 'Fettarme Lebensmittel – Multiverfahren zur Gaschromatographischen Bestimmung von Pestizidrückständen – Teil 2: Verfahren zur Extraktion und Reinigung, Verfahren N', Deutsches Institut für Normung, Berlin (1998).
7. W. Specht, S. Pelz, and W. Gilsbach, *Fresenius' J. Anal. Chem.,* **353**, 183 (1995).
8. G. Kempe, E.-M. Kschonek, and K. Speer, 'Assessment of the DFG Method S19-Online for Residue Analysis of Azole Pesticides,' Presented at the 3rd European Pesticide Residue Workshop, York, UK, July 3–5, 2000.
9. Deutsche Forschungsgemeinschaft, 'Rückstandanalytik von Pflanzenschutzmitteln, Stand der 11. Lieferung,' VCH, Weinheim, Aufarbeitungsverfahren XII-5 (1991).
10. W. Ernst, R.G. Schaefer, H. Goerke, and G. Eder, *Fresenius' J. Anal. Chem.,* **272**, 358 (1974).

Neonicotinoids

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1 Introduction

Neonicotinoids are potent broad-spectrum insecticides that exhibit contact, stomach and systemic activity. Acetamiprid, imidacloprid, nitenpyram, thiamethoxam and thiacloprid are representatives of the neonicotinoid insecticides (Figure 1). The mechanism of action is similar to that of nicotine, acting on the central nervous system causing irreversible blocking of postsynaptic nicotinic acetylcholine receptors (nAChR). Neonicotinoid insecticides are often categorized as antagonists of the nAChR.[1–4]

Neonicotinoids are generally polar, nonvolatile crystals with high water solubility. They are nonionized at environmentally relevant pH and are stable to heat and sunlight. Neonicotinoids are stable to acid hydrolysis. Except for thiacloprid, they are susceptible to alkaline hydrolysis.

Tolerances for pesticide residue and/or standard withholding registration of neonicotinoids in Japan are shown in Table 1.

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Residue analytical methods for neonicotinoids in crops, soil and water samples have been developed. The basic principle of these methods consists of the following steps: extraction of the crop and/or soil samples with acetone or the other organic solvent, cleanup by liquid–liquid partition or column chromatography, and quantitative analysis by high-performance liquid chromatography with ultraviolet detection (HPLC/UV). Simple column cleanup procedures are used to improve the accuracy and sensitivity of these methods.

2 Analytical methodology for plant materials

2.1 Nature of the residue

In general, neonicotinoids (except for nitenpyram) are metabolized slowly in plants, and remain mainly as the parent compounds. The definition of crop residues is for the acetamiprid and imidacloprid parent molecule. The definition of crop

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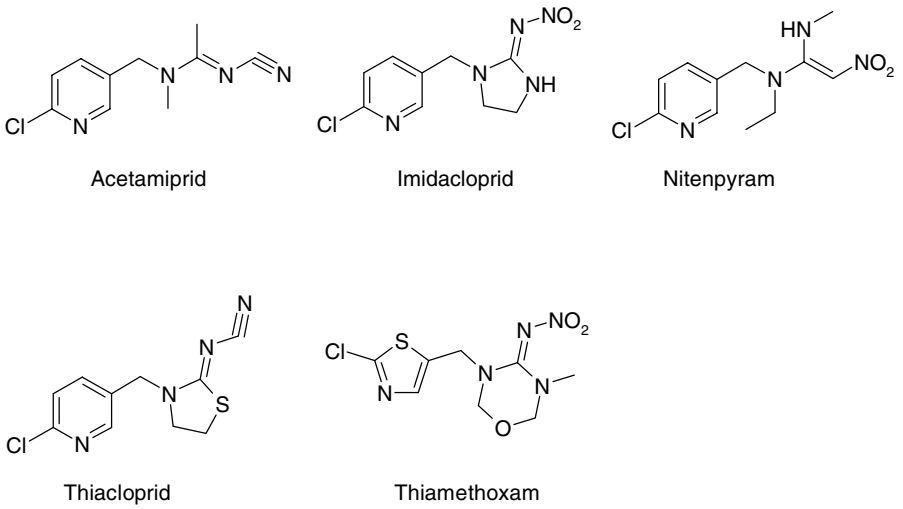


Figure 1 Structures of neonicotinoid insecticides

residues for nitenpyram includes both the parent and the following metabolites: 2-[*N*-(6-chloro-3-pyridyl-methyl)-*N*-ethyl]amino-2-methyliminoacetic acid (CPMA) and *N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl-*N′*-methylformamidine (CPMF). Since both CPMA and CPMF are unstable during the analytical process, they are derivaterized to the corresponding formamide, *N*-(6-chloro-3-pyridylmethyl)-*N*-ethylformamide (CPF), for gas chromatography (GC) determination (Figure 2).[5]

The definition of crop residues for thiamethoxam and thiacloprid includes the parent and its metabolite: *N*-(2-chlorothiazol-5-ylmethyl)-*N′*-methyl-*N″*-nitroguanidine (designated the guanidine compound) for thiamethoxam, and 3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylideneaminocarboxamide (designated the amide compound) for thiacloprid[6] (Figure 3).

Table 1 Tolerances for pesticide residues and/or standard withholding registration of neonicotinoids

	Tolerance for pesticide residue and/or standard withholding registration (mg kg^{-1} or mg L^{-1})				
Crop	Acetamiprid	Imidacloprid	Nitenpyram	Thiacloprid	Thiamethoxam
Vegetables	1–5	5	5	1–5	0.5
Tea	50	5	10	25	
Potato, radish (root), sweet potato	0.5	0.1	0.2	0.1	0.5
Rice	—	0.2	0.5	0.1	0.1
Fruits	5	0.3–3	0.5	1–5	0.5
Tomato, eggplant, cucumber	5	1	5	1	0.5
Water	—	2	13	0.3	0.5

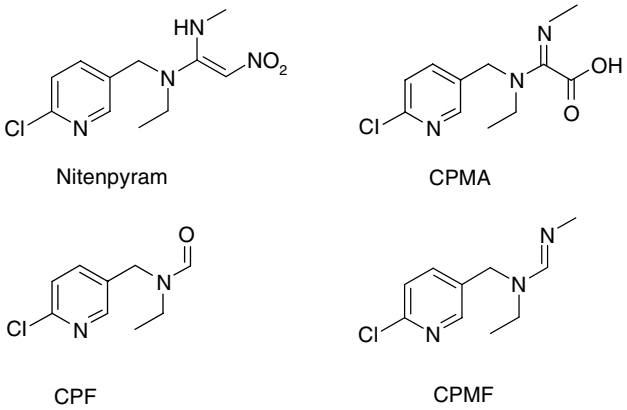


Figure 2 Possible metabolites of nitenpyram in plants and chemically derived products (CPF)

2.2 Analytical method principle

The general outline of the high-performance liquid chromatography (HPLC) method for neonicotinoids is as follows. Homogenized samples such as fruits and vegetables are extracted with acetone. In the case of rice grain, samples are added to water and allowed to stand for 2 h, then extracted with organic solvent. After evaporation of the acetone extract, the aqueous phase is transferred into a macroporous diatomaceous column. Neonicotinoids are eluted with ethyl acetate from the column. The eluate is evaporated to dryness in vacuum and the residue is dissolved in n-hexane, which is subjected to a cleanup procedure using a Florisil or silica gel cartridge. The concentrated eluate is determined by HPLC. Specific details on the extraction, cleanup and chromatographic determination are given in the following sections.

DropBooks

2.2.1 Preparation of crop samples

Crop samples, 0.5–2 kg, are chopped into small pieces and homogenized thoroughly in a food processor. A typical analytical sample size is <50 g. To prevent the potential degradation of the analytes during sample storage, samples should be frozen immediately after collection and maintained frozen until analyzed.

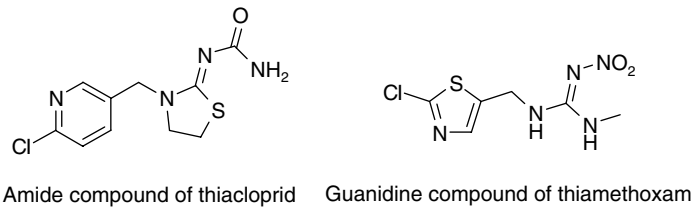


Figure 3 Structures of metabolites of thiacloprid and thiamethoxam

2.2.2 Extraction

(1) Vegetables and fruits. A 20-g sample of the homogenized vegetable or fruit sample is weighed into an Erlenmeyer flask. After adding 100 mL of acetone (acetonitrile is used to extract imidacloprid samples), the mixture is shaken vigorously on a mechanical shaker for 30 min, then filtered under vacuum through a filter paper, which is covered with a glass filter paper. The residue is transferred into the flask to repeat the same procedures as before, i.e., addition of acetone (50 mL), shaking and filtration. The filtrates are combined and concentrated to approximately 20 mL with a rotary evaporator.[7,8]

Alba *et al.*[9] used ethyl acetate to extract imidacloprid residues from fruits and vegetables. A 15-g sample of vegetable or fruit is weighed into a blender tube and 60 mL of ethyl acetate and 15 g of sodium sulfate are added. The mixture is homogenized for 30 s, using a Polytron, and filtered. The filtrate is evaporated and the residue obtained is dissolved in acetonitrile–water (1 : 1, v/v). Alba *et al.*[9] considered the low recoveries of these polar pesticides as the major disadvantage of the acetone extraction method. In their previous work they evaluated the efficacy of ethyl acetate for the extraction of pesticide residues.

(2) Brown rice, wheat and bean. Several analytical procedures have been developed for rice grain. In the case of rice straw, finely cut samples are added to water and allowed to stand for 2 h, then extracted with acetone. Unpolished rice grain samples are milled with an ultracentrifuge mill and sieved through a 42-mesh screen prior to extraction.

A 10-g sample of the homogenized sample is weighed into an Erlenmeyer flask and soaked in 20 mL of distilled water for 2 h. After adding 100 mL of acetone and shaking the mixture vigorously in a mechanical shaker for 30 min, the extract is filtered. The same acetone (50 mL) extraction procedure is repeated. The filtrates are combined and acetone is removed with a rotary evaporator.[5]

(3) Tea. A 4-g sample is homogenized and soaked with 16 mL of distilled water for 2 h. Methanol (100 mL) is added to the mixture and shaken for 30 min. The extract is filtered through a Celite layer (1–2 cm thickness) under vacuum. The filter cake and vessel are washed twice, each with 25 mL of methanol. The combined filtrates are transferred to a separatory funnel.[10]

2.2.3 Cleanup procedure

The extent of the cleanup depends on the sample matrix to be analyzed, the extraction procedure, the method of detection and the desired sensitivity. Generally, the cleanup method is liquid–liquid partitioning (LLP), but recently it has become simpler and more reliable to use solid-phase extraction (SPE) columns.

(1) LLP

(a) Organic solvent partition. A 150-mL volume of 5% sodium chloride solution is added to the filtrate derived from Section 2.2.2. The solution is shaken twice,

each with 100 mL of n-hexane, for 10 min. The n-hexane layer is discarded and the aqueous layer is shaken twice, each with 100 mL of dichloromethane, for 10 min. The combined dichloromethane phase is passed through a filter paper with anhydrous sodium sulfate, and concentrated.

(b) Acetonitrile–n-hexane partition. For rice grain and nut samples, an additional cleanup step is required to remove oily residues in the crop sample extract prior to GC or HPLC analysis. Techniques such as acetonitrile–n-hexane partitioning after extraction or a cleanup procedure such as LLP, gel permeation chromatography (GPC), a macroporous diatomaceous earth column (e.g., Chem Elut column) or a C_{18} cartridge, etc., are used. Acetonitrile–n-hexane partitioning is found to be an effective procedure for removing oily materials.

(2) Column chromatography

(a) Macroporous diatomaceous column (e.g., Chem Elut Column). The LLP step described above can be replaced by using a Chem Elut column. The sample extract is concentrated to 20 mL and applied directly to a Chem Elut column at room temperature for 5–10 min. Using the Chem Elut column, imidacloprid is eluted with 80 mL of dichloromethane after washing the column with 60 mL of n-hexane. Acetamiprid and thiamethoxam are eluted with 120–140 mL of ethyl acetate after washing with the same volume of ethyl acetate.[6,8] Nitenpyram is eluted with 50 mL of dichloromethane after washing with 50 mL of n-hexane and 50 mL of diethyl ether–hexane (1 : 1) successively.[5]

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(b) SPE column (e.g. silica gel and Florisil cartridge). Examples of cleanup procedures using silica gel and Florisil cartridge columns are described as follows. In the case of acetamiprid, the eluate from the Chem Elut column described above is evaporated and cleaned up with a Florisil cartridge. The Florisil cartridge is rinsed with 15 mL of n-hexane and acetamiprid is eluted with 40 mL of n-hexane–acetone (7 : 3, v/v). The final residue is dissolved in a suitable volume of water–acetonitrile (4 : 1, v/v) and analyzed by HPLC (246 nm).

For imidacloprid, the combined filtrates are concentrated with a rotary evaporator. The final residue is dissolved in 5 mL of n-hexane and applied to a silica gel cartridge, preconditioned with 5 mL of n-hexane. The n-hexane solution is transferred into the cartridge, which is rinsed with 5 mL of n-hexane–ethyl acetate (1 : 1, v/v) and eluted with 8 mL of ethyl acetate. Further cleanup could be carried out using a Florisil cartridge, if needed. The imidacloprid residue in 4 mL of n-hexane–acetone (13 : 7, v/v) is eluted with 8 mL of n-hexane–acetone (2 : 3, v/v). The eluate is concentrated under vacuum on a rotary evaporator at 40 °C. The residue obtained is dissolved in a suitable volume of water–acetonitrile (4 : 1, v/v) and analyzed by HPLC (270 nm).[8]

There is a reported example of using an open silica gel column for the purification of nitenpyram. The extract containing nitenpyram is evaporated and applied to a silica gel column (silica gel 10 g and anhydrous sodium sulfate 5 g). Nitenpyram is eluted

with 50 mL of acetone after washing the column with 50 mL of dichloromethane–acetone (1 : 1, v/v). The eluate is concentrated and the residue is dissolved in 5 mL of 0.05 M monobasic potassium phosphate–methanol (17 : 3, v/v) and analyzed by HPLC.[5]

(c) Polystyrene (PS)–cation-exchange cartridges. The concentrated sample extract is added to 10 mL of water and applied to a PS cartridge (500 mg, ENVI-Chrom P or GL-Pak PLS-2) preconditioned with 5 mL each of methanol and water. To this PS cartridge, an ion-exchange column such as SCX is connected. Nitenpyram is eluted from the PS cartridge with 10 mL of water–methanol (1 : 1, v/v) and the eluate is applied to an SCX cartridge. Nitenpyram is eluted with 10 mL of methanol–ammonia solution (99 : 1, v/v) after washing the SCX cartridge with 10 mL of methanol. The eluate is concentrated at 40 °C, the final residue is applied to a neutral alumina cartridge and nitenpyram is eluted with 20 mL of methanol.[5]

(d) Cation-exchange cartridge and alumina-N cartridge. Thiamethoxam and its metabolite, N-(2-chlorothiazol-5-ylmethyl)-N′-methyl- N″-nitroguanidine, are eluted with 10 mL of methanol from SCX without using a PS cartridge, and then applied directly to an alumina-N cartridge. The analytes are eluted with 20 mL of acetone after washing the alumina N-cartridge with 5 mL of acetone.[6]

2.2.4 Determination

Several determination methods such as GC, HPLC, gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) are used for the analysis of neonicotinoid residues. The applications of GC/MS and LC/MS are of increasing importance. The application of HPLC to the determination of neonicotinoids residues is limited, especially when metabolites (such as acetamiprid and nitenpyram) can be easily determined by GC after derivatization.

(1) HPLC and LC/MS. HPLC methodology coupled with ultraviolet (UV), fluorescence (FL), photodiode-array (PDA) and/or a mass spectrometry (MS) detection has been developed. In general, neonicotinoids can be determined by HPLC/UV. Typical HPLC operating conditions are given in Table 2.

(2) GC. Typical GC operating conditions are given in Table 3.

(3) Enzyme-linked immunosorbent assay (ELISA). Li and Li[11] developed an ELISA procedure for imidacloprid to determine its residues in coffee cherry and bean extracts. A 25-g amount of sample extracted with 300 mL of methanol and 1% sulfuric acid (3 : 1, v/v) for 3 min. An aliquot of the sample extract (0.5 mL) is mixed with 1 mL of water and a gentle stream of nitrogen is used to evaporate methanol. The solution is then extracted with 1 mL of ethyl acetate, the extract is reconstituted in 1 mL of PBST (phosphate-buffered saline containing 0.05% Tween 20) and competitive ELISA is performed to quantify imidacloprid in the extract. For methanol extracts of coffee cherries and beans fortified with imidacloprid at 0.5 mg L^{-1}, recoveries of imidacloprid by the ELISA method were 108 and 94, respectively.

Table 2 HPLC and LC/MS operating conditions for the determination for neonicotinoids

	Acetamiprid	Imidacloprid	Nitenpyram	Thiacloprid	Thiamethoxam	Imidacloprid
Analyte	Acetamiprid	Imidacloprid	Nitenpyram metabolite [a]	Thiacloprid metabolite [b]	Thiamethoxam metabolite [c]	Imidacloprid
Instrument	HPLC	HPLC	HPLC	HPLC	HPLC	LC/APCI-MS
Detection[d]	UV	PAD	UV	UV	UV	MS
Wavelength (nm)	246	270	270	240	258	m/z 256;
Column:	L-Column	LiChrospher-C18	HRC-ODS	Inertsil ODS	L-Column	Zorbax SB-C8
i.d. (mm)	4.6	4	4.6	4.6	4.6	4.6
Length (cm)	25	12.5	25	25	25	15
Particle size (μm)	5	5	5	5	5	3.5
Temperature (°C): column oven	40	40	40	40	40	40
Mobile phase (v/v)[e]	ACN–water (1 : 4)	ACN–water (25 : 75 to 100 : 0)	0.05 M KH_2PO_4 MeOH (17 : 3)	ACN–water parent (2 : 3) Met (1 : 4)	ACN–water (3 : 17)	ACN–50 mM $HCOONH_4$ (1 : 19) (0 : 100 to 100 : 0)
Flow rate of mobile phase (mL min^{-1})	1	1	1	1	1	1
Retention time (min)	15.6	3.5	9.2 (parent) 7.9 (CPMA) 5.3 (CPMF)	9 (parent) 13 (Met)	15 (parent) 22.5 (Met)	9.5
Reference	Ueji *et al.*[8]	Alba *et al.*[12]	Tsumura *et al.*[5]	Personal data	Personal data	Alba *et al.*[9]

[a] 2-[*N*-(6-Chloro-3-pyridylmethyl)-*N*-ethyl]amino-2-methyliminoacetic acid (CPMA) and *N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl-*N*′-methylformamidine (CPMF).
[b] Amido compound.
[c] Guanidine compound.
[d] PAD, photodiode-array detection.
[e] ACN, acetonitrile; Met, metabolite.

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Table 3 GC operating conditions for the determination for neonicotinoids

	Acetamiprid	Acetamiprid	Nitenpyram
Analyte	Acetamiprid	Acetamiprid metabolite[a]	Nitenpyram and metabolite[b]
Detection	NPD	ECD	FTD
Column:	DB-17	DB-17	DB-17
i.d. (mm)	0.53	2	0.25
Length (m)	10	2.1	30
Film thickness (μm)	1	1	0.25
Temperature (°C):			
Column oven	100 °C (1 min), 30 °C min^{-1}, 270 °C (3 min)	125	50 °C (2 min), 10 °C min^{-1}, 250 °C
Injection	260	250	270
Detector	280	250	270
Flow rate of gas (mL min^{-1}):			
Carrier gas (N_2)		0.5	
Carrier gas (He)	10		1.2
Makeup gas (N_2)			
Hydrogen	3		
Air	100		
Retention time (min)	7	8	20 (CPF)
Reference	Personal data	Tokieda *et al.*[10] (total residue method)	Tsumura *et al.*[5] (total residue method)

[a] 2-(2,4-Dichloro-3-methylphenoxy)propionic acid (DMPA).
[b] 2-[*N*-(6-Chloro-3-pyridylmethyl)-*N*-ethyl]amino-2-methyliminoacetic acid (CPMA) and *N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl-*N′*-methylformamidine (CPMF), *N*-(6-chloro-3-pyridymethyl)-*N*-ethylformamide (CPF).

2.2.5 Evaluation

Quantitation is performed by the calibration technique. A new calibration curve with neonicotinoid standard solutions is constructed for each set of analyses. The peak area or peak height is plotted against the injected amount of neonicotinoid. The injection volume (2 μL) should be kept constant as the peak area or peak height varies with the injection volume. Before each set of measurements, the GC or HPLC system is calibrated by injecting more than one standard solution containing ca 0.05–2 ng of neonicotinoid. Recommendation: after constructing the calibration curve in advance, standard solutions and sample solutions are alternately injected for measurement of actual samples.

2.2.6 Recoveries, limit of detection (LOD) and limit of quantitation (LOQ)

The LOD is an important criterion of the efficiency of an analytical method. It defines the smallest value of the concentration of a compound in the analytical sample. Detectable amounts of neonicotinoid insecticides range from 0.5 to 1 ng by HPLC. The LOQ ranges from 0.005 to 0.01 mg kg^{-1} for vegetables, fruits and crops.

The acceptable method recoveries using untreated plant matrices at fortification levels between 10 and 50 times the LOD must be from 70 to 120%. The relative standard deviation (RSD) must be within the range 10–20% (according to the analytical method of the Ministry of the Environment, Japan).

HPLC determination can be carried out for most of the neonicotinoids. The average recoveries of imidacloprid in the various crops by the HPLC/PDA method were 88–94% at a fortification level of 0.25 mg kg^{-1} and 96–99% at a fortification level of 0.05 mg kg^{-1}. The overall average recovery for 30 samples was 95% with an RSD of 4.7%.[12]

According to the official Japanese HPLC/UV method, the average recovery for grapes fortified with 0.2 mg kg^{-1} of acetamiprid was 96% and that for tomato fortified with 0.4 mg kg^{-1} of imidacloprid was 90% (personal data). The recovery of nitenpyram from fruits, vegetables and rice was 66–85% at 0.2–0.8 mg kg^{-1} fortification levels. The LOD was 0.0025–0.01 mg kg^{-1}.[5]

LC/MS is used as a multi-residue analytical method. The recovery of imidacloprid from tomato was 90–105% for 0.05 and 0.5 mg kg^{-1}. The LOD for imidacloprid was <10 μg kg^{-1} in the full-scan mode and 1 μg kg^{-1} in the selected-ion monitoring (SIM) mode.[9]

Using the gas chromatography/electron capture detection (GC/ECD) method, the average recovery of acetamiprid from fruits and vegetables fortified at 0.1 mg kg^{-1} was 96% and the LOD was 0.005 mg kg^{-1}. For green tea, the recovery of acetamiprid fortified at 0.05 mg kg^{-1} ranged from 95 to 98%. As described in detail in Section 2.2.8, the recoveries of acetamiprid and its metabolites, IM-2-1, IM-0, IC-0 and IM-0-glucose, in crops ranged from 74 to 92% by the total residue determination method using GC/ECD. The LOD was 0.01 mg kg^{-1}.[10,13] The recoveries of nitenpyram and its metabolites, CPMA and CPMF (0.5–2.0 mg kg^{-1} fortification levels), from fruits, vegetables and rice were 64–120%. The LOD was 0.0025–0.01 mg kg^{-1}.[5]

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2.2.7 Calculation of residues

The amount of neonicotinoids insecticide residue (R, mg kg^{-1}) in the sample is calculated by the following equation:

$$R = (W_i / V_i) \times (V_f / G)$$

where

G = sample weight (g)
V_i = injection volume into gas chromatograph or high-performance liquid chromatograph (μL)
V_f = final sample volume (mL)
W_i = amount of neonicotinoid insecticide for V_i read from calibration curve (ng)

2.2.8 Other analytical methods

(1) Acetamiprid and its metabolites. A GC method has been developed for the determination of acetamiprid and its metabolites IM-2-1, IM-0, IC-0 (Figure 4) and IM-0-glucose in crops. As shown in Figure 5, acetamiprid and its metabolites in crops are extracted with methanol and derivatized to methyl 6-chloronicotinate (IC-0-Me) through alkaline hydrolysis, potassium permanganate oxidation and then esterification

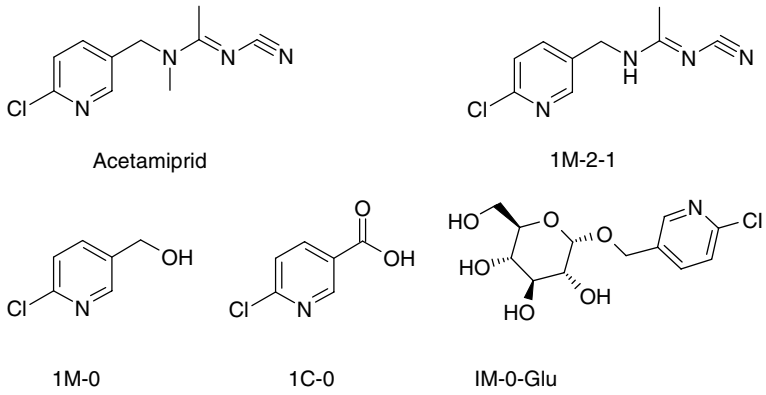


Figure 4 Possible metabolites of acetamiprid in plants

by diazomethane, followed by column chromatography cleanup and GC determination. The LOD is 0.01 mg kg^{-1} and the recoveries of fortified samples ranged from 74 to 92%.[13]

(2) Nitenpyram and its metabolites. The metabolites of nitenpyram, CPMA and CPMF, are determined by HPLC under the same conditions as for the parent nitenpyram. The retention times of nitenpyram, CPMA, and CPMF are 9.2, 7.9 and 5.3 min, respectively.[5] However, these compounds are unstable and need to be derivatized to a more stable compound, CPF, prior to analysis. It is necessary to remove acetone from the extract before derivatization, because a by-product can be formed in the presence of acetone thus impacting the recovery of CPF. Nitenpyram is more effectively determined using HPLC, whereas CPF, as the analyte of nitenpyram and its metabolites, is more effective by gas chromatography/flame thermionic detection (GC/FTD).

The residue analysis of CPMA and CPMF in vegetables and fruits is carried out as follows. A 20-g amount of the sample is homogenized for 3 min with 100 mL of acetone and filtered. The extraction procedure is repeated once with 50 mL of acetone and filtered. The combined filtrate is concentrated to 10 mL at 50 °C and, after addition of 0.2 mL of triethylamine to the concentrate, the reaction mixture is allowed to stand for 30 min at 50 °C. The mixture is applied to an Extrelut column (Extrelut 14-g). CPF from CPMA via CPMF is eluted with 50 ml of diethyl ether after washing the

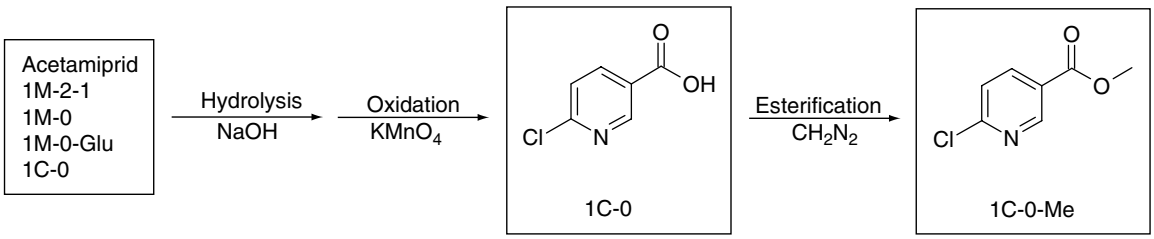


Figure 5 Derivatization reaction of acetamiprid and its metabolites

column with 50 mL of hexane. The eluate is concentrated and subjected to silica gel chromatography (silica gel 10 g and anhydrous sodium sulfate 5 g). CPF is eluted with 50 mL of acetone after washing the column with 50 mL of acetone–hexane (1 : 4, v/v). The eluate is concentrated and the residue is dissolved in a suitable volume of acetone. The solution is injected into the GC/FTD system. Recoveries from fruits, vegetables, rice and tea were 66–85% for nitenpyram (0.2–0.8 mg kg^{-1} fortification levels) and 64–120% for metabolites (0.5–2.0 mg kg^{-1} fortification levels); recovery data for CPMA in tea are not available. Detection limits were 0.0025–0.01 mg kg^{-1} for nitenpyram and 0.025–0.1 mg kg^{-1} for the metabolites.[5]

2.2.9 Important points in analysis

(1) Analytical procedure. Homogenization and milling for rice grain and rice straw samples must be carried out in the presence of dry-ice. During evaporation of organic solvents, the temperature of the water bath should be kept at 40 °C or lower.

(2) Extraction efficiency. The efficiency of extractions of imidacloprid from vegetables and crops decreases in the order acetonitrile > acetone > ethyl acetate. Acetonitrile is not a suitable extraction solvent because of the large number of co-eluting peaks on the chromatograms on HPLC at 210 or 270 nm.[12]

Extraction of neonicotinoid from crop materials is often performed using the classical methods, which include the coagulation of the oily material on a solid support prior to extraction.

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(3) Peak type on chromatogram. The shape of the matrix peaks depends on the nature of the sample and also on the organic mobile phase content. For HPLC, since low-level detection is required, the interference of co-extract materials in the samples should be minimized.

3 Analytical methodology for soil

3.1 Nature of soil residues

The polar character of neonicotinoids makes them, in general, potentially mobile in soil. Acetamiprid and nitenpyram have short soil persistence. Imidacloprid and thiamethoxam, however, are sufficiently persistent in soil to be used for soil treatment. The definition of soil residues for the various neonicotinoid compounds except for imidacloprid are the parent compound and it metabolites. The metabolites of acetamiprid are 1M-1-2, 1M-1-4 and 1C-0 (Figure 6). The metabolites of nitenpyram are 2-[*N*-(6-chloro-3-pyridyl-methyl)-*N*-ethyl]amino-2-methyliminoacetic acid (CPMA) and *N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl-*N′*-methylformamidine] (CPMF).

Two residue analytical methods have been developed for acetamiprid: one method determines the parent acetamiprid only and the other determines by GC the total content of acetamiprid and its degradation products.[13–15] A similar method is also used for nitenpyram.[5]

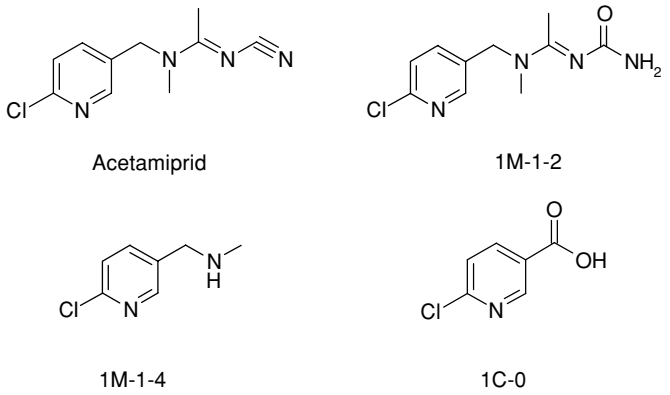


Figure 6 Possible metabolites of acetamiprid in soil

3.2 Analytical method

3.2.1 Preparation of analytical samples

In the laboratory, field samples should be mixed thoroughly. The air-dried soils are passed through a 2-mm sieve to remove stones and roots, then the water content of the soil is calculated after drying at 105 °C for 5 h. If the samples cannot be analyzed immediately after drying and sieving, they should be stored at about −20 °C in glass or Teflon bottles fitted with screw-caps.

3.2.2 Extraction

Extraction of neonicotinoid residues from soil is much more difficult than their extraction from plant or water samples. Soil residues could exist as 'bound residue'. Various extraction methods such as organic solvent extraction, supercritical fluid extraction (SFE), Soxhlet extraction and sonication have been used. Some extraction methods are described in the following.

(1) Organic solvent extraction. Two analytical methods for acetamiprid have been developed: One method is for the parent only and the other determines the total residue of the parent and its metabolites (1M-1-2, 1M-1-4 and 1C-0).[15] Air-dried soil (20-g equivalent dry soil) is weighed into a centrifuge tube and imidacloprid residue is extracted with 100 mL of methanol–0.1 M ammonium chloride (4 : 1, v/v) using a mechanical shaker for about 30 min. After shaking, the tube is centrifuged at 8000 rpm for 2 min. The supernatant is filtered and the analysis of the soil residue is carried out in the same manner as described above for the parent compound.

The second acetamiprid extraction method uses aqueous methanol, and with alkaline methanol to extract acetamiprid and its degradation products which are converted to methyl 6-chloronicotinate (IC-0-Me) through alkaline hydrolysis, oxidation and esterification, prior to column cleanup and GC determination.

The extraction method for imidacloprid residues has been presented by Westwood *et al.*[16] A 20-g soil sample is extracted with 20 mL of acetonitrile–water (4 : 1, v/v) by

shaking overnight. After centrifugation and evaporation, the final residue is dissolved in acetonitrile and analyzed by HPLC.

(2) SFE. SFE has been established as the extraction method of choice for solid samples. The usefulness of SFE for soil samples has been demonstrated for carbamate,[17] organophosphorus and organochlorine pesticides.[18] However, SFE is more effective in extracting nonpolar than polar residues. In order to obtain a greater extraction efficiency for the polar residues of imidacloprid, the addition of 20% methanol as modifier is required. Extraction at 276 bar and 80 °C with a solvent consisting of supercritical carbon dioxide modified with methanol (5%) for 40 min gives a recovery of 97% (RSD = 3.6%, $n = 10$). It is possible to use process-scale SFE to decontaminate pesticide residues from dust waste.[19]

3.2.3 Cleanup procedure

(1) Macroporous diatomaceous column (e.g., Chem Elut column). The combined soil extract is concentrated to dryness under vacuum, the residue is dissolved in 15 mL of water and the solution is applied to a Chem Elut column. After charging for 20 min, acetamiprid is eluted with 100 mL of dichloromethane. The eluate is evaporated to dryness under vacuum.[15]

(2) SPE column: C_{18} cartridge. The concentrated eluate from the Chem Elut column as described above is dissolved in 5 mL of distilled water and charged on a C_{18} cartridge pretreated with 20 mL each of methanol and distilled water. Acetamiprid is eluted with the 30 mL of 15% acetonitrile solution and the eluate is collected and concentrated to dryness at 40–50 °C under vacuum. The residue is dissolved in a suitable volume of acetone and analyzed by GC.[15]

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3.2.4 Determination

The determination procedure is described in Section 2.2.4.

3.2.5 Evaluation

The evaluation procedure is described in Section 2.2.5.

3.2.6 Recoveries, limit of detection and limit of quantitation

The LOD for the HPLC method for acetamiprid and its degradation products (IM-1-2, IM-1-4 and IC-0) in soil is 0.01 mg kg^{-1}. The recoveries of these compounds at a fortification level of 0.1 mg kg^{-1} ranged from 70 to 95%.

In the GC method, the recoveries of acetamiprid and its degradation products in soil are >95% by the individual method for the parent compound (parent determination method). On the other hand, the recovery ranged from 74 to 96% by the total residue determination method with a limit of detection of 0.01 mg kg^{-1}.[15]

Eskilsson and Mathiasson[19] reported a 97% recovery of imidacloprid from dust waste by the SFE method.

3.2.7 Calculation of residues

Calculation of residues in soil is described in Section 2.2.7.

3.2.8 Other soil analytical methods

(1) Acetamiprid and its degradation products. The total residue method is also applicable to the determination of acetamiprid and its degradation products in soil. A 20-g soil sample (dry weight base) is extracted with 100 mL of methanol–0.1 M ammonium chloride (4 : 1, v/v) for 30 min. Methanol–0.5 M NaOH (4 : 1, v/v) is added to the soil for the second extraction. A 100-mL volume of water is added to the combined supernatant, and rinsed with 100 mL of n-hexane. The aqueous layer is concentrated to 20 mL, 30 mL of water and 0.6 g of NaOH are added to the concentrate and then the solution is stirred for 2 h at 95 °C. After the hydrolysis, an oxidation reaction is carried out by adding 1 g of potassium permanganate to the reaction mixture and heating at 95 °C for 30 min. The reaction solution is adjusted to pH 1.5 with hydrochloric acid and extracted with 150 mL of dichloromethane–acetone (1 : 1, v/v). The extract is concentrated, the residue is dissolved in 5 mL of methanol, and 5 mL of diazomethane in diethyl ether are added. The mixture is kept for 30 min at room temperature. After esterification, 10 mL of methanol and 20 mL of distilled water are added and the mixture is extracted three times with 20 mL each of n-hexane for 5 min. The n-hexane layer is concentrated and cleaned up on a silica gel (10-g) column. 6-Chloronicotinate (IC-0-Me) is eluted with 130 mL of the same solvent mixture after rinsing with 110 mL of diethyl ether–n-hexane (1 : 49, v/v). The eluate is concentrated, dissolved in n-hexane and analyzed by GC. The amounts of IC-0-Me obtained are calculated relative to the equivalent amount of parent acetamiprid by applying a factor of 1.30 (ratio of molecular weight of acetamiprid to that of IC-0-Me).[15]

3.2.9 Important points

(1) Extraction. Centrifuging the soil extract in the screw-capped vial can easily break the solvent emulsions that often form during extraction. The vial can survive up to 6000 *g* centrifugation if rubber stoppers are inserted into the centrifuge cup to provide a flat base to protect the vials. The desired phase (usually the upper) can be easily removed with a pipet or, if it is to be discarded, it can be removed using a disposable pipet connected by tubing to a suction flask and a vacuum line.

4 Analytical methodology for water

4.1 Nature of the residues

The definition of water residue for the neonicotinoid insecticides except for nitenpyram and thiacloprid is the parent molecule. For nitenpytram and thiacloprid both the parent and its metabolites are determined. These metabolites are 2-[*N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl]amino-2-methyliminoacetic acid (CPMA) and

N-(6-chloro-3-pyridylmethyl)-*N*-ethyl-*N*′-methylformamidine] (CPMF) for nitenpyram and 3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylideneaminocarboxamide (designated the amide compound) for thiacloprid.[6]

4.2 Analytical method

4.2.1 Sample preparation

Water samples should be extracted immediately after collection or after arrival in the laboratory. They should be stored at 5 °C until analysis.

4.2.2 Extraction

Extraction methods include solvent extraction, SPE using a cartridge or disk, a solid-phase microextraction (SPME), etc.

(1) Solvent extraction. Extraction of neonicotinoid insecticides from water is a simple process involving saturation with sodium chloride and extraction with diethyl ether, dichloromethane or ethyl acetate. This extraction procedure will allow the simultaneous extraction of all neonicotinoids.

A 10-g sample of sodium chloride and 50 mL of dichloromethane are added to the water sample (200 mL) and the mixture is shaken vigorously using a mechanical shaker for 5 min at room temperature. The dichloromethane layer is separated and the aqueous layer is extracted again with 50 mL of dichloromethane. The combined dichloromethane layer is dried with anhydrous sodium sulfate.

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(2) SPE cartridge. In a recent method, the water sample is extracted using an SPE column such as C_{18} or a PS-2 cartridge with an extraction machine. This procedure is simple and rapid.

Shimamura *et al.*[20] developed a monitoring method for 22 pesticides (including imidacloprid) in river water. The method is based on the liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) determination after extraction by styrene–divinylbenzene copolymer (Waters Sep-Pak Plus PS-2 cartridges, containing 265 mg of the copolymer) using an auto-concentrator. The recoveries of 22 pesticides fortified at the 10 or 30 $\mu g\,L^{-1}$ levels ranged from 81 to 103%. The recovery of imidacloprid ranged from 93 to 103% at a fortification level of 0.03 $mg\,L^{-1}$. The LOD was 0.02–0.3 $\mu g\,L^{-1}$.

Other extraction methods use an SPE disk and SPME. The analysis of water samples using SPE disks (SPE-C_{18} disk) was performed according to the methodology described by Albanis and Hela.[21] Generally, the SPME method is a more reliable technique than SPE for trace analysis that can shorten the analytical procedure.

4.2.3 Determination

The determination procedure is described in Section 2.2.4. The ELISA determination of water samples can be performed directly, such as for imidacloprid in tap water.[11]

4.2.4 Evaluation

The evaluation procedure is described in Section 2.2.5.

References

1. I. Yamamoto, G. Yabuta, M. Tomizawa, T. Saito, T. Miyamoto, and S. Kagabu, *J. Pestic. Sci.,* **20**, 33 (1995).
2. M. Tomizawa, H. Otsuka, T. Miyamoto, M.E. Elderfrawi, I. Yamamoto, and G. Yabuta, *J. Pestic. Sci.,* **20**, 57 (1995).
3. M. Tomizawa, H. Otsuka, T. Miyamoto, and I. Yamamoto, *J. Pestic. Sci.,* **20**, 49 (1995).
4. I. Minamida, K. Iwanaga, T. Tabuchi, I. Aoki, T. Fusaka, H. Ishizuka, and T. Okauchi, *J. Pestic. Sci.,* **18**, 41 (1993).
5. Y. Tsumura, Y. Nakamura,Y. Tonogai, Y. Kakimoto, Y. Tanaka, and T. Shibata, *Shokuhin Eiseigaku Zasshi,* **39**, 127 (1998).
6. Ministry of the Environment, 'The Official Analytical Method for Thiamethoxam of the Ministry of the Environment, Japan,' Ministry of the Environment, Tokyo (2001) (in Japanese).
7. Ministry of Health, Welfare and Labor, 'The Official Analytical Method for Acetamiprid of the Ministry of Health, Welfare and Labor, Japan,' Ministry of Health, Welfare and Labor, Tokyo (1997) (in Japanese).
8. M. Ueji, H. Kobayashi, and K. Nakamura, '2002 Version Analytical Methods of Pesticides,' Soft Science, Tokyo, pp. 25, 306, 383, 482 (2001) (in Japanese).
9. A.R.F. Alba, A. Tejedor, A. Agüera , M. Contreras, and J. Garrido, *J. AOAC Int.,* **83**, 748 (2000).
10. M. Tokieda, K. Iiyoshi, K. Sugioka, and T. Gomyo, *J. Pestic. Sci.,* **22**, 129 (1997).
11. K. Li and Q.X. Li, *J. Agric. Food Chem.,* **48**, 3378 (2000).
12. A.R.F. Alba, A. Valverde, A. Agüera, M. Contreras, and S. Chiron, *J. Chromatogr. A,* **721**, 97 (1996).
13. M. Tokieda, M. Ozawa, S. Kobayashi, and T. Gomyo, *J. Pestic. Sci.,* **22**, 77 (1997).
14. M. Tokieda, T. Tanaka, M. Ozawa, and T. Gomyo, *J. Pestic. Sci.,* **23**, 296 (1998).
15. M. Tokieda, M. Ozawa, and T. Gomyo, *J. Pestic. Sci.,* **24**, 181 (1999).
16. F. Westwood, K.M. Bean, A.M. Dewar, R.H. Bromilow, and K. Chamberlain, *Pestic. Sci.,* **52**, 97 (1998).
17. A. Izquierdo, M. T. Tena, M.D. Luque de Castro, and M. Valcarcel, *Chromatographia,* **42**, 206 (1996).
18. J.L. Synder, R.L. Grob, M.E. McNally, and T.S. Oostdyk, *J. Chromatogr. Sci.,* **31**, 183 (1993).
19. C.S. Eskilsson and L. Mathiasson, *J. Agric. Food Chem.,* **48**, 5159 (2000).
20. Y. Shimamura, N. Tomiyama, M. Murakoshi, H. Kobayashi, and O. Matano, *J. Pestic. Sci.,* **23**, 241 (1998).
21. T.A. Albanis and D.G. Hela, *J. Chromatogr.,* **707**, 283 (1995).

Oxime carbamates

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1 Introduction

Oxime carbamates are *N*-methylcarbamate pesticides which were first introduced in the 1960s and have found a wide range of uses in the treatment of seed, soil, and crops. At present, oxime carbamates are used worldwide as insecticides, acaricides, nematicides, and molluscicides.[1] These compounds exhibit high insect toxicity, moderate to high mammalian toxicity via the oral route, and relatively short persistence after application. Their mode of action is based on the inhibition of the enzyme acetylcholinesterase in the transmission of impulses in the nervous system.[2] Oxime carbamates form a reversible complex with acetylcholinesterase which may result in the blockage of nerve signals, causing paralysis or death of the pest species. Entry of the insecticides to the target site is through the cuticle (contact) or by ingestion. The acute toxicity of oxime carbamates ranges from moderately to highly toxic.[1] Table 1 shows the chemical names, structures, and properties of representative oxime carbamates.

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Oxime carbamates have high polarity and solubility in water and are relatively chemically and thermally unstable. They are relatively stable in weakly acidic to neutral media (pH 4–6) but unstable in strongly acidic and basic media. Rapid hydrolysis occurs in strongly basic aqueous solutions (pH > 9) to form the parent oxime/alcohol and methylamine, which is enhanced at elevated temperature. Additionally, oxime carbamates are, generally, stable in most organic solvents and readily soluble in acetone, methanol, acetonitrile, and ethyl acetate, with the exception of aliphatic hydrocarbons. Furthermore, most oxime carbamates contain an active *S*-alkyl (methyl) moiety that can be easily oxidized to form the corresponding sulfoxide or sulfone metabolites.

Oxime carbamates are not directly amenable to gas chromatography (GC) because of their high thermal instability, which often leads to their breakdown at the injection port or in the column during analysis. Analysis of oxime carbamates by GC with sulfur detection or flame photometric detection involves oxidation of the intact insecticides[3] or alkaline hydrolysis to form the more volatile but stable oxime compound.[4] Enzymatic techniques have been reported for the analysis of these compounds. Enzyme-linked immunosorbent assay (ELISA) has been used to determine aldicarb and its sulfone and sulfoxide metabolites[5] and methomyl in water, soil, and sediment samples.

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Table 1 Chemical names and properties of selected oxime carbamates

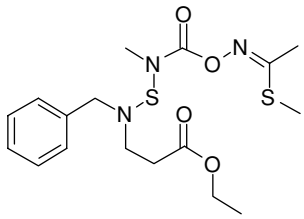
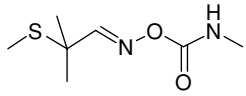
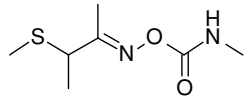
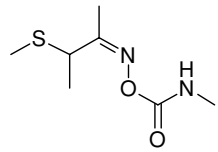
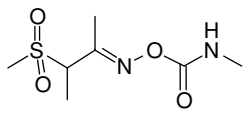
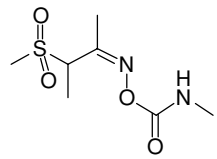
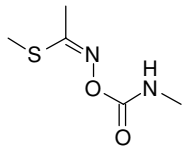
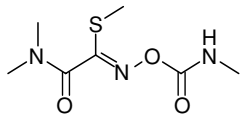
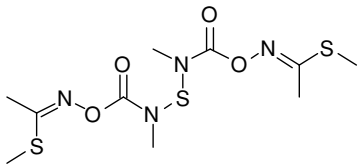
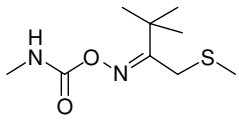
Chemical name and properties	Structure
Alanycarb (insecticide) IUPAC name: ethyl (Z)-*N*-benzyl-*N*-[[methyl(1-methylthioethylideneamino-oxycarbonyl)amino]thio]-β-alaninate CA name: (Z)-ethyl 3,7-dimethyl-6-oxo-9-(phenylmethyl)-5-oxa-2,8-dithia-4,7,9-triazadodec-3-en-12-oate CAS RN: [83130-01-2] Water solubility and vapor pressure: 0.020 g L^{-1} (20 °C), <0.0047 mPa (20 °C) LD_{50} for rats (oral): 440 mg kg^{-1}	
Aldicarb (insecticide, acaricide, nematicide) IUPAC name: 2-methyl-2-(methylthio)propionaldehyde *O*-methylcarbamoyloxime CA name: 2-methyl-2-(methylthio)propanal *O*-[(methylamino)carbonyl]oxime CAS RN: [116-06-3] Water solubility and vapor pressure: 4.93 g L^{-1} (20 °C), 13 mPa (20 °C) LD_{50} for rats (oral): 0.93 mg kg^{-1}	
Butocarboxim (insecticide) IUPAC name: 3-(methylthio)butanone *O*-methylcarbamoyloxime CA name: 3-(methylthio)-2-butanone *O*-[(methylamino)carbonyl]oxime CAS RN: [34681-10-2] Water solubility and vapor pressure: 35 g L^{-1} (20 °C), 10.6 mPa (20 °C) LD_{50} for rats (oral): 153–215 mg kg^{-1}	 (*E*)  (*Z*)
Butoxycarboxim (insecticide, acaricide) IUPAC name: 3-methylsulfonylbutanone *O*-methylcarbamoyloxime CA name: 3-(methylsulfonyl)-2-butanone *O*-[(methylamino)carbonyl]oxime CAS RN: [34681-23-7] Water solubility and vapor pressure: 209 g L^{-1} (20 °C), 0.266 mPa (20 °C) LD_{50} for rats (oral): 458 mg kg^{-1}	 (*E*)  (*Z*)
Methomyl (insecticide, acaricide) IUPAC name: *S*-methyl *N*-(methylcarbamoyloxy)thioacetimidate CA name: methyl *N*-[[(methylamino)carbonyl]oxy]ethanimidothioate CAS RN: [16752-77-5] Water solubility and vapor pressure: 57.9 g L^{-1} (25 °C), 6.65 mPa (25 °C) LD_{50} for rats (oral): 17–24 mg kg^{-1}	
Oxamyl (insecticide, acaricide, nematicide) IUPAC name: *N*,*N*-dimethyl-2-methylcarbamoyloxyimino-2-(methylthio)acetamide CA name: methyl 2-(dimethylamino)-*N*-[[methylamino)carbonyl]oxy]-2-oxoethanimidothioate CAS RN: [23135-22-0] Water solubility and vapor pressure: 280 g L^{-1} (25 °C), 31 mPa (25 °C) LD_{50} for rats (oral): 5.4 mg kg^{-1}	

Table 1—*Continued*

Thiodicarb (insecticide, molluscicide) IUPAC name: 3,7,9,13-tetramethyl-5,11-dioxa-2,8,14-trithia-4,7,9,12-tetraazapentadeca-3,12-diene-6,10-dione CA name: dimethyl *N*,*N*′-[thiobis[(methylimino)carbonyloxy]]bis(ethanimidothioate) CAS RN: [59669-26-0] Water solubility and vapor pressure: 35 mg L^{-1} (25 °C), 5.7 mPa (20 °C) LD_{50} for rats (oral): 66 mg kg^{-1}	
Thiofanox (insecticide, acaricide) IUPAC name: 3,3-dimethyl-1-methylthiobutanone *O*-methylcarbamoyloxime CA name: 3,3-dimethyl-1-(methylthio)-2-butanone *O*-[(methylamino)carbonyl]oxime CAS RN: [39196-18-4] Water solubility and vapor pressure: 5.2 g L^{-1} (22 °C), 22.6 mPa (25 °C) LD_{50} for rats (oral): 8.5 mg kg^{-1}	

The most common methods for the quantitative analyses of oxime carbamates and their metabolites involve reversed-phase high-performance liquid chromatography (HPLC). Ultraviolet (UV) absorbance can be employed as a detection method;[5,6] however, these compounds do not contain, generally, a strong UV-absorbing chromophore that allows selective and high-sensitivity detection. Strong absorption maxima of these compounds normally occur in the UV region at 202 nm or less,[7] where plant co-extractives also commonly show strong absorption. The detection method that is most commonly used is fluorescence in conjunction with derivatization reactions since oxime carbamates do not possess native fluorescence properties. The analytes separated through the HPLC column are hydrolyzed at elevated temperature with sodium hydroxide or in an anion-exchange resin (Aminex A-27, catalyst) to form the methylamines, which are then derivatized with *o*-phthaldehyde and 2-mercaptoethanol or *N*,*N*-dimethyl-2-mercaptoethylamine forming highly fluorescent isoindole derivatives, and their fluorescence is measured in an on-line detector.[8–28]

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More recent methods for the quantitative analysis of oxime carbamates in various matrices use reversed-phase HPLC with mass spectrometry (MS) and tandem mass spectrometry (MS/MS) for detection. Early applications of HPLC with MS detection involved a moving belt interface[29] and thermospray ionization.[30,31] With the introduction of the atmospheric pressure ionization (API) systems with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces, determination of oxime carbamates by high-performance liquid chromatography/mass spectrometry (HPLC/MS) became widespread.[32–35] Analysis of oxime carbamates by high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) using a triple-quadrupole mass spectrometer with a positive-ion electrospray interface has also grown.[36] HPLC/MS/MS is more sensitive and specific than HPLC/MS and allows the simultaneous detection and confirmation of the compounds being analyzed.

The analytical methods summarized in this article are generally multiresidue methods for the determination of oxime carbamates in different sample matrices (crops, animal tissues, soil, and water). These methods include HPLC with fluorescence, MS, and MS/MS detection.

2 Analytical methodology

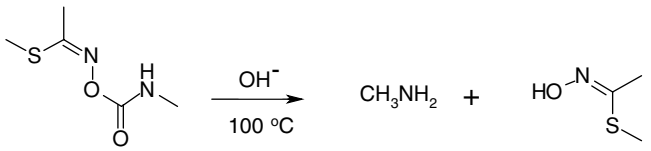
Reversed-phase HPLC followed by post-column derivatization and subsequent fluorescence detection is the most common technique for quantitative determination of oxime carbamate insecticides in biological and environmental samples. However, for fast, sensitive, and specific analysis of biological and environmental samples, detection by MS and MS/MS is preferred over fluorescence detection. Thus, descriptions and recommendations for establishing and optimizing HPLC fluorescence, HPLC/MS, and HPLC/MS/MS analyses are discussed first. This is followed by specific rationales for methods and descriptions of the recommended residue methods that are applicable to most oxime carbamates in plant, animal tissue, soil, and water matrices.

2.1 Reversed-phase HPLC/fluorescence analysis

The most common approach for detection and quantitation of oxime carbamates and their metabolites in biological and environmental matrices is by reversed-phase HPLC followed by post-column derivatization and subsequent fluorescence detection. HPLC analyses are carried out using C_{18}, C_8, or phenyl silica-based columns and mobile phase consisting of water and an organic solvent such as acetonitrile and methanol. Post-column derivatization is a two-stage process that converts the oxime carbamates in the column effluent into a highly fluorescent derivative. The first stage is the hydrolysis of the molecule at elevated temperature by sodium hydroxide (NaOH)[9,18–25,27–30] or in an anion-exchange resin (Aminex A-27, catalyst)[8,10,11,26] to release methylamine. The second stage is the derivatization of the released methylamine with OPA and 2-mercaptoethanol in borate solution or OPA and *N*,*N*-dimethyl-2-mercaptoethylamine (Thiofluor) to produce a fluorophore compound of substituted isoindole.

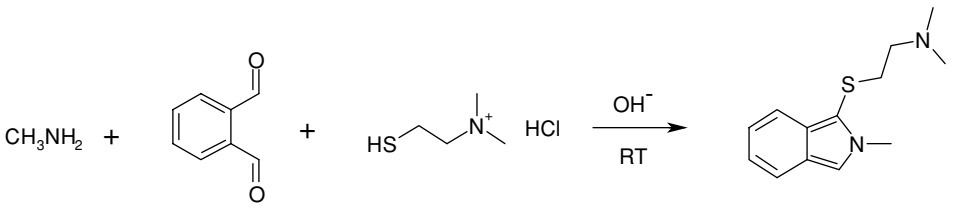
The post-column reactions of methomyl are illustrated in Equations (1) and (2).

Hydrolysis:



Methomyl Methylamine Oxime (1)

Derivatization:



o-Phthalaldehyde Thiofluor™ 1-Dimethylaminoethylthio-2-methylisoindole (2)

For HPLC/fluorescence analysis, an HPLC system capable of performing linear gradients at constant flow rate is adequate. Using a chilled auto sampler maintained at 4 °C to minimize the degradation of samples while in queue for analysis is recommended. Also, a thermostated column compartment is recommended to enable chromatographic separations to be performed at a fixed temperature so as to minimize variability in retention time and to obtain a consistent response. A complete post-column HPLC system specific for the determination of *N*-methylcarbamates by hydrolysis with NaOH and derivatization by OPA is commercially available (Pickering Model PCX-5200, Pickering Laboratories, Mountain View, CA, USA). All solvents used should be of HPLC grade and degassed. Furthermore, the fluorescence detector should be capable of excitation at 330 nm and detection of emission energies above 418 nm. Descriptions of the apparatus and reagents for post-column hydrolysis and derivatization reactions and the chromatographic conditions used for the analysis of methomyl in crops by DuPont Crop Protection and Batelle, Geneva Research Centres, are given in Table 2.

2.2 *Reversed-phase HPLC/MS and HPLC/MS/MS analysis*

The recommended technique for the determination of oxime carbamates and their metabolites by HPLC/MS and HPLC/MS/MS is positive ESI. Electrospray is a soft ionization technique and is suitable for thermally labile compounds. Ions are produced in the liquid phase at quasi-ambient temperature and atmospheric pressure, thus leaving the fragile pesticides intact. For oxime carbamates, the molecular adducts that can be monitored during HPLC/MS analysis with electrospray in positive mode are $[M+H]^+$, $[M+Na]^+$, or $[M+NH_4]^+$, depending on the nature of mobile phase used.[33–35]

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For HPLC/MS analysis, a bench-top single-quadrupole mass spectrometer with an electrospray interface is sufficient. Optimization of the response of an individual analyte via flow injection analysis or infusion is done by injecting an aqueous solution of the analyte directly into the electrospray ion source without a column present. The concentration of the analyte is usually at the micrograms per milliliter level (e.g., 1–5 $\mu g\ mL^{-1}$), and the mobile phase composition and flow rate should approximately match (within ~25%) the elution conditions of the analyte from the analytical column. Typically, a 1 : 1 (v/v) water–organic solvent composition of mobile phase and a flow rate of 0.2–0.3 $mL\ min^{-1}$ represent a good starting point. The MS parameters, such as drying and nebulizer nitrogen flow rates, drying temperature, fragmenter voltage, etc., are optimized to provide maximum response for the $[M+H]^+$ ion or that molecular adduct that gives a relatively high abundance. Evaluation of spectral information can be obtained in full-scan mode. However, detection and quantitation of oxime carbamates are achieved by selected ion monitoring (SIM), which offers greater sensitivity and selectivity than the full-scan mode. In the SIM mode, data are collected only at the *m/z* (charge-to-mass ratio) of ions of analytical interest. The *m/z* selected should be abundant and unique, i.e., the ion should not be common in the matrix or background.

For HPLC/MS/MS analysis, a triple-quadrupole mass spectrometer with an electrospray interface is recommended for achieving the best sensitivity and specificity in the quantitative determination of oxime carbamates and their metabolites. This allows

the monitoring of two parent-to-daughter transitions, which can be used to confirm the presence of analytes in a sample and reduces the risk of false positive detections. Moreover, owing to the sensitivity and specificity of this technique, this eliminates the need for excessive cleanup. The HPLC/MS/MS determination of oxamyl

Table 2 HPLC/fluorescence analysis of methomyl in crops (Multi-residue Method 2)

(A) Equipment	
HPLC/fluorescence instrument	
HPLC pump	Waters 600E Multisolvent Delivery System
Injector	Waters Model 717 Plus (thermostated)
Temperature controller module	Waters CHM for column heating
Data system	Waters Millennium version 3.20
Fluorescence detector	Waters 474 tunable fluorescence detector
Reagent pump (post-column):[a]	Merck-Hitachi 655A-13, equipped with one reactor containing a 10-m Teflon loop (thermostated at 95 °C) for hydrolysis which is connected to a 50-μL Teflon loop (at ambient temperature) for fluorescence reagent addition
Heating bath with heating fluid circulation	Type DT-1 (Heto, Allerod, Denmark)
Flow rate	0.25 mL min^{-1}

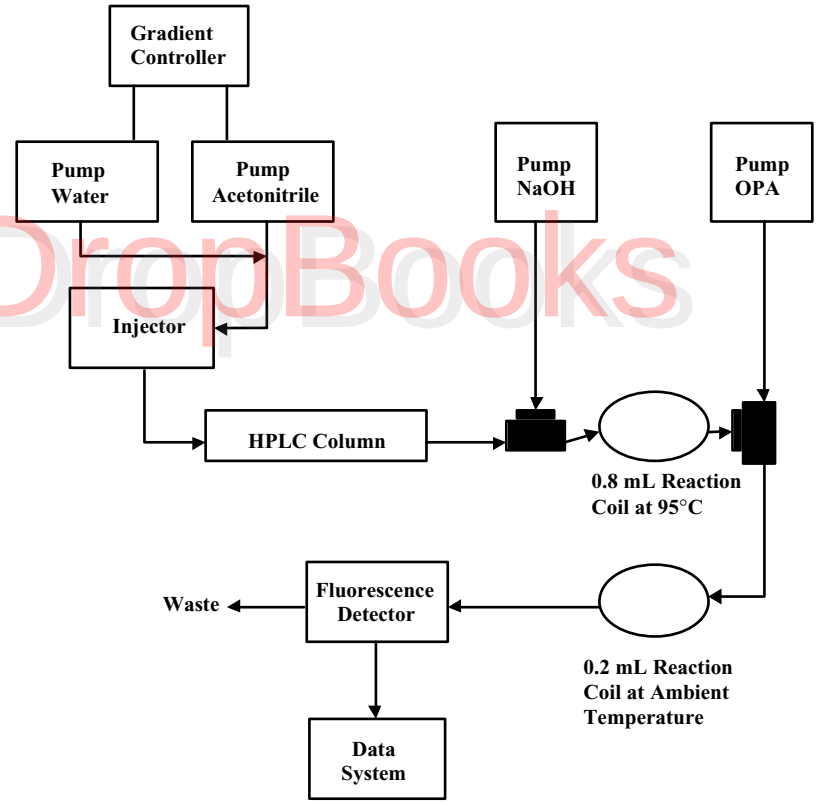
(B) Reagents/solutions
Acetone, HPLC grade, 99.8%
Acetonitrile, HPLC grade, 99.9%
1-Decanol, 99.5%
Dicloromethane, 99.8%
Fluoraldehyde (Pierce Fluorescence Reagent, contents 0.8 mg mL^{-1} highly purified fluoraldehyde phthaldehyde crystals, Brij-35 and mercaptoethanol in specially formulated borate buffer
Hexane, 99%
Methanol, HPLC grade
Sodium chloride, reagent grade
Petroleum ether, b.p. 40–60 °C
Ultrapure water
1% 1-decanol (keeper): dilute 1 mL of 1-decanol with dichloromethane to 100 mL
0.2 M sodium hydroxide (aq.): dissolve the appropriate amount of sodium hydroxide pellets in ultrapure water. Filter the solution under vacuum using a 0.45-mm disk filter. Prepare the solution fresh every 2 days
Fluorescence reagent for post-column derivatization: a 4-fold dilution of Pierce Fluorescence reagent prepared in ultrapure water
0.05 μg mL^{-1} Trimethacarb solution (internal standard): dilute a 5 μg mL^{-1} trimethacarb solution in acetonitrile with water–acetonitrile (13 : 7, v/v) 100-fold. Prepare the solution fresh every 2 days

(C) HPLC conditions	
Column	Zorbax C_8, 250 × 4.6-mm i.d., 5.0-μm particle size
Column temperature	30 °C
Autosampler temperature (recommended)	15 °C (4 °C)
Injection volume	80 μL
Mobile phase conditions and flow rate	Solvent A: water

Table 2—*Continued*

	Solvent B: acetonitrile		
	Time (min)	% A	% B	Flow rate (mL min^{-1})
	0.00	75	25	1.00
	7.00	75	25	1.00
	8.00	50	50	1.00
	16.00	50	50	1.00
	17.00	75	25	1.00
	22.00	75	25	1.00
Fluorescence detector excitation and emission wavelengths	330 and 466 nm, respectively		
Bandwidth	18		
Filter type	RC		

(D) Block diagram of the HPLC/fluorescence system



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[a] A complete post-column LC system for the analysis of oxime carbamates using this approach is commercially available (Pickering Laboratories). Alternative post-column hydrolysis conditions: 50 × 4.0-mm i.d., 15 µm, Aminex A-27 column (Bio-Rad), 120 °C.

and its oxime metabolite in crops, soil, and water matrices has been conducted in positive ion mode with water and methanol as HPLC mobile phases. Formation of the gas-phase ions of either $[M + H]^+$ or $[M + NH_4]^+$ (in the presence of NH_4^+ in the mobile phase) under these conditions is favorable, and fragmentation into two daughter ions is possible. Although the $[M + Na]^+$ ions of oxamyl and other oxime carbamates are, in some cases, more abundant than the $[M + H]^+$ ions, they are not desirable because they are difficult to fragment further into daughter ions. Table 3 provides an example of HPLC/MS/MS conditions used by DuPont Crop Protection for the determination of oxamyl in drinking and surface waters. In this example, aqueous samples (100-μL) are injected with a weak mobile phase composition followed by gradient elution for the separation of oxamyl from the matrix components. Oxamyl

Table 3 HPLC/MS/MS conditions for the determination of oxamyl in drinking and surface waters

HPLC system	Agilent HP1100 HPLC				
Column	Phenomenex Luna phenyl-hexyl, 150 × 4.6-mm i.d., 3.0-μm particle size				
Column temperature	40 °C				
Autosampler temperature	4 °C				
Injection volume	100 μL				
Mobile phase condition	Time (min)	% A	% B	Flow rate (mL min^{-1})	
	0.00	80	20	1.0	
	6.00	50	50	1.0	Solvent A: water
	7.00	30	70	1.0	Solvent B: methanol
	7.50	0	100	1.0	
	8.50	0	100	1.0	
	9.00	80	20	1.0	
	11.00	80	20	1.0	
Oxamyl retention time	~6.0 min				
MS/MS system	Micromass Quattro II (triple quadrupole)				
Analyte	Ions monitored (*m/z*)	Cone voltage (V)	Collision energy (V)	Dwell time (s)	Acquisition[a] timing (min)
Oxamyl	220.0 → 71.8 ± 0.1	30	10	0.25	5.0–7.5
	220.0 → 89.9 ± 0.1	30	11	0.25	5.0–7.5
Ion mode	ESI positive				
Electrospray voltage	4.1 kV				
Detector voltage	700 V				
Source heater	150 °C				
Collision gas pressure	2.5×10^{-3} mbar				
Nebulising gas flow rate	15 L h^{-1}				
Drying gas flow rate	300 L h^{-1}				
MS flow rate (post-column split):	100 μL min^{-1} (approximately 10 : 1 split)				

[a] Before and after the acquisition time, the effluent flow is directed to waste to minimize MS source contamination.

is eluted in about 6 min followed by the cleaning and re-equilibration periods. The MS data collection is between 5 and 7.5 min. A six-port electronically activated switching valve is used to divert the HPLC effluent to waste before and after the collection window in order to reduce ion source contamination. Since the electrospray interface performs optimally at low flow rates, the HPLC effluent flow is split such that approximately 100 $\mu L\,min^{-1}$ actually passes through the interface (~10:1 split) while the remainder goes to a waste container.

Optimization of the MS/MS response is typically done by infusing directly an aqueous solution of each analyte into the electrospray ion source. The composition of the analyte's solution and/or mobile phase and flow rate should approximately match the conditions expected at the time of the analyte's elution. The instrument is first operated in the MS mode, and the settings for the electrospray ion source are optimized to provide maximum response for the $[M + H]^+$ ion or that molecular adduct which is relatively abundant, e.g., $[M + NH_4]^+$. This is followed by the optimization of the collision cell to produce maximum response of two parent-to-daughter transition ions. Monitoring at least two transition ions per analyte during the course of analysis is recommended. With two parent-to-daughter transition ions monitored or multiple reaction monitoring (MRM), the identity of the analyte detected can be verified, especially when there are contamination issues. The identity of the analyte is confirmed when the ratio of the signals for the MRM transitions obtained for the sample matches that of a calibration standard within ±30%.[37] However, for controlled studies, when the history of the analyte's application to the sample is known, one parent-to-daughter ion transition is usually considered sufficient to confirm the analyte's presence. The control sample will be used to demonstrate that baseline interference is less than a signal-to-noise ratio of 3 : 1.

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HPLC/MS and HPLC/MS/MS analyses are susceptible to matrix effects, either signal enhancement or suppression, and are often encountered when the cleanup process is not sufficient. To assess whether matrix effects influence the recovery of analytes, a post-extraction fortified sample (fortified extract of control sample that is purified and prepared in the same manner as with the other samples) should be included in each analytical set. The response of the post-extraction fortified sample is assessed against that of standards and samples. Matrix effects can be reduced or corrected for by dilution of samples, additional cleanup, or using calibration standards in the sample matrix for quantitation.

A sample set for HPLC/MS, HPLC/MS/MS, or HPLC/fluorescence analysis should have at least four chromatographic standards prepared at concentrations equivalent to 50–70% of the limit of quantitation (LOQ) up to the maximum levels of analytes expected in the samples. These standards should be prepared in similar solvent used to prepare the fortified and investigative samples in a set for chromatographic analysis. The sample set should also have at least two control samples fortified at the LOQ and at 10 times the LOQ or at the expected highest residue concentration of investigative samples to verify the method performance. The first injection should be that of a chromatographic standard or reagent blank for system equilibration, followed by the lowest standard, then by at least 3–4 control, fortified, or investigative samples, followed by another chromatographic standard. This sequence is repeated until all the samples have been injected. The last injection should be that of a standard. In order to minimize the degradation of analytes during the analysis, samples and standards

should be prepared in a weakly acidic aqueous solution (pH 4–5) with an organic modifier of methanol or acetonitrile. Moreover, the use of a chilled autosampler maintained at 4 °C prevents further degradation of analytes and samples. (Note: for samples containing methomyl, alanycarb, and thiodicarb, it is recommended that the samples and their extracts should be kept on dry-ice even when being actively processed.[38] Alanycarb and thiodicarb are unstable in various solvent extracts at ambient temperature and may degrade rapidly to methomyl.)

Calibration curves generated for each analyte from the chromatographic standards should be linear (correlation coefficient $R > 0.99$) with negligible intercepts so that either linear regression or a response factor method may be used for residue calculations.

2.3 Crops, food, feed, and animal tissue

2.3.1 Nature of the residue

Oxime carbamates are generally applied either directly to the tilled soil or sprayed on crops. One of the advantages of oxime carbamates is their short persistence on plants. They are readily degraded into their metabolites shortly after application. However, some of these metabolites have insecticidal properties even more potent than those of the parent compound. For example, the oxidative product of aldicarb is aldicarb sulfoxide, which is observed to be 10–20 times more active as a cholinesterase inhibitor than aldicarb.[1] Other oxime carbamates (e.g., methomyl) have degradates which show no insecticidal activity, have low to negligible ecotoxicity and mammalian toxicity relative to the parent, and are normally nondetectable in crops. Therefore, the residue definition may include the parent oxime carbamate (e.g., methomyl) or parent and metabolites (e.g., aldicarb and its sulfoxide and sulfone metabolites). The tolerance or maximum residue limit (MRL) of pesticides on any food commodity is based on the highest residue concentration detected on mature crops at harvest or the LOQ of the method submitted for enforcement purposes if no detectable residues are found. For example, the tolerances of methomyl in US food commodities range from 0.1 to 6 mg kg^{-1} for food items and up to 40 mg kg^{-1} for feed items.[39]

2.3.2 Rationale for methods

Oxime carbamates and their metabolites are efficiently extracted from watery, dry, and oily crop matrices by homogenization or blending the matrices with organic solvents. Various extraction solvents of different polarities have been used, e.g., polar, water-miscible organic solvents, such as acetone,[3,8] acetonitrile,[12,13] and methanol,[14–16] or water-immiscible solvents, such as ethyl acetate.[4] For dry crop matrices (<75% water), the sample is sometimes soaked in water prior to homogenization in an organic solvent to extract residues effectively. Another extraction procedure that has been used is matrix-solid phase dispersion (MSPD), which includes sample homogenization, extraction, and purification in the same process.[17] The sample is homogenized with a derivatized silica sorbent, which is placed in a column, and the analytes are selectively eluted with organic solvents. Another extraction technique is accelerated solvent

extraction (ASE), which involves sample homogenization with hydromatrix or silica and extraction at high temperature and pressure.[18] ASE achieves rapid extraction with small volumes of conventional organic solvents.

A cleanup procedure is usually carried out to remove co-extracted matrix components that may interfere in the chromatographic analysis or be detrimental to the analytical instrument. The cleanup procedure is dependent on the nature of the analyte, the type of sample to be analyzed, and the selectivity and sensitivity of the analytical instrument used in the analysis. Preliminary purification of the sample extracts prior to chromatographic separation involves liquid–liquid partitioning and/or solid-phase extraction (SPE) using charcoal/Celite, Florisil, carbon black, silica, or aminopropyl-silica based adsorbents or gel permeation chromatography (GPC).

Separation of the very polar, water-soluble crop co-extractives, e.g., sugars, is achieved by partitioning the extracted analytes into water-immiscible (low-polarity) organic solvents such as dichloromethane or petroleum ether.[8–11] In other methods, the analytes are directly partitioned into the organic solvent by saturation of the aqueous extract with sodium chloride.[14,15] For the removal of nonpolar co-extractives such as fats and oils, either aqueous or organic sample extracts are partitioned with hexane.[4] Purification by column chromatography or SPE separates the analytes from either polar and/or nonpolar co-extractives depending on the class of adsorbents and type of loading and eluting solvents used.[8–25] A GPC cleanup separates the analytes from the high molecular weight crop co-extractives, such as lipids, fats, and oils, which are excluded from the column and are first to elute.[19,20]

The purified sample extracts are concentrated and analyzed by reversed-phase HPLC with fluorescence, MS, or MS/MS detection as described in Sections 2.1 and 2.2.

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2.3.3 Description of methods

(1) Crops, food, and feed. The first recommended method is based on the Dutch Multiresidue Method 2[8] for *N*-methylcarbamates, which was originally developed by de Kok *et al.*[9–11] The method has recently been validated by DuPont Crop Protection and Batelle, Geneva Research Centres, for the analysis of methomyl and oxamyl in dry, high-water, high-fat, and high-acid content crops and in various grape processed products. The limit of detection for each analyte is 0.003–0.005 mg kg^{-1}.

A 15-g amount of a well-mixed chopped crop sample is weighed in a 250-mL polypropylene centrifuge bottle. The sample is fortified, if necessary, by pipetting 100–500 μL of the appropriate standard in acetonitrile on to the sample before any extracting solution is added and then allowing the sample to air-dry for 15 min. Dry and watery crop samples are extracted by homogenization [using a Polytron PTA20 SM (Brinkman Instrument, Westbury, NY, USA) or equivalent] in 30 mL of acetone for 30 s. (For dry crops, soaking the samples in 10 mL of water, without stirring, for 10 min before homogenization in acetone is recommended in order to hydrate the matrix for a better extraction. For liquid matrices, using 40 mL of acetone and homogenizing by mechanical shaking for 15 min is recommended.) A 30-mL volume of dichloromethane and 30 mL of petroleum ether are added, and the sample is homogenized for another 60 s. After homogenization, the sample is centrifuged for 2 min at 4000–6000 rpm, and the upper (organic extract) layer is decanted into an Erlenmeyer

flask. A 2-mL aliquot of the organic extract is transferred to a 10-mL glass tube (this represents an aliquoting factor of 50). The extract is evaporated to near dryness under a gentle stream of dry nitrogen at 40–60 °C (N-Evap, Organomation Associates, South Berlin, MA, USA) and the remaining solvent is allowed to evaporate in the air. The residue is reconstituted in 1 mL of dichloromethane. A Bond Elut aminopropyl SPE cartridge (100-mg) (Varian, Harbor City, CA, USA) is conditioned with 1 mL of dichloromethane, and the cartridge is never allowed to go dry after conditioning. The 1-mL dichloromethane sample extract is applied to the cartridge, followed by the 0.5 mL of dichloromethane which is used for rinsing the sample tube. The eluate is collected in a 10-mL glass tube immediately after applying the extract. Elution is continued with 1 mL of dichloromethane–methanol (99:1) solvent mixture, and the eluate is collected in the same tube. A 50-μL volume of 20% ethylene glycol in acetone (or 5 drops of 1% decanol) is added as a keeper, the eluate is evaporated to near dryness under a gentle stream of dry nitrogen at 40–50 °C, and the remaining solvent is allowed to evaporate in the air. The residue is reconstituted in 1 mL of 0.05 $\mu g\,mL^{-1}$ trimethacarb solution (internal standard) in an acetonitrile–water mixture equivalent to the HPLC mobile phase solvent with the help of an ultrasonic bath (1-min). The sample solution is filtered through a 0.2- or 0.45-μm PTFE filter for HPLC fluorescence analysis.

For oily samples, e.g., nutmeats, a 15-g sample should be homogenized in 100 mL of acetonitrile for 2 min, followed by the addition of 5 g of sodium chloride (NaCl) and homogenization for another 30 s. Following centrifugation, the layers are allowed to separate for at least 30 min, and 20 mL of the upper acetonitrile layer are pipetted into a 125-mL separatory funnel. The organic extract is partially purified by shaking the extract three times with 20 mL of hexane for 30 s, and the hexane (top layer) is discarded after every shaking. The acetonitrile extract (bottom layer) is drained into a 50-mL glass tube, and a 2-mL aliquot is transferred into a 10-mL glass tube. For the subsequent cleanup of the acetonitrile extract (from the oily sample) and preparation for HPLC analysis, the same procedures as used for the dry and watery crop samples are followed.

If the analytes of interest are not quantitatively recovered from the above method (such is the case for aldicab sulfoxide), the method by Fillion *et al.*[12] is recommended. The method uses acetonitrile for extraction followed by a salting-out step and sequential purifications of the acetonitrile extract using octadecyl (C_{18}), carbon, and aminopropyl SPE cartridges. In this case, 50 g of sample are homogenized in 100 mL of acetonitrile at half-speed for 5 min. About 10 g of NaCl are added to induce phase separation, and the sample is homogenized for another 5 min. The sample is centrifuged, and the aqueous and acetonitrile phases are allowed to separate. A Bond Elut C_{18} SPE cartridge (1-g/6-mL) (Varian) is preconditioned with acetonitrile and conditioned further with 2 mL of the acetonitrile extract (top layer), which is discarded. The cartridge should not be allowed to go dry after conditioning. A 15-mL volume of the acetonitrile extract is applied to the cartridge and eluted by gravity flow into a 15-mL centrifuge tube. Eluate collection is stopped when the volume in the collection tube reaches 13 mL. Anhydrous sodium sulfate (Na_2SO_4) is added to bring the liquid to 15 mL, and the tube is capped and shaken well. After centrifugation at high speed for 5 min, a 10-mL aliquot (equivalent to a 5-g sample) of extract is transferred to another 15-mL centrifuge tube and evaporated to 0.5 mL under nitrogen

at 35 °C. A Sep-Pak aminopropyl cartridge (360-mg) (Waters, Milford, MA, USA) is attached to the bottom of an Envi-Carb cartridge (500-mg) (Supelco, Bellefonte, PA, USA), and both cartridges are conditioned with acetonitrile–toluene (3 : 1) solution. The concentrated extract is quantitatively transferred to a carbon cartridge by rinsing the centrifuge tube with acetonitrile–toluene (3 : 1), eluted through the stacked cartridges, and elution is completed with 20 mL of acetonitrile–toluene (3 : 1) solution. The eluate is collected immediately after applying the extract. The eluate is evaporated to low volume using a rotary evaporator with the water-bath set at 35 °C. Two 10-mL portions of acetone are added, and the sample is evaporated to a low volume after each addition to make a solvent exchange to acetone. The extract is quantitatively transferred to a 15-mL graduated centrifuge tube and evaporated under nitrogen at 35 °C to make a solvent exchange to 0.8 mL of pH 3 water. A 20-μL volume of the isoprocarb internal standard solution (40-μg mL^{-1}, 1.0-ng mL^{-1} final concentration) is added to the sample solution. The final sample should be filtered through a 0.2-μm PTFE filter prior to HPLC fluorescence analysis.

(2) Animal tissue (cattle, swine, and poultry liver). The recommended multiresidue method for oxime carbamates in animal tissue is the method of Ali.[21] The LOQ for oxime carbamates, specifically aldicarb and its sulfone and sulfoxide metabolites and methomyl, in beef, pork, and duck liver is 5 μg kg^{-1}. Partially defrosted processed liver (21 g) is weighed into a 500-mL homogenizer flask [VirTis flask (American Scientific Products, Stone Mountain, GA, USA) or equivalent]. The sample is fortified if necessary. Anhydrous Na_2SO_4 (60 g) is added to the sample and mixed with a spatula, followed by 200 mL of dichloromethane and mixed with the same spatula. The sample mixture is homogenized (VirTis 45 homogenizer or equivalent) for 2 min at medium speed, and the clear extract is suction filtered through a 9-cm i.d. Buchner funnel containing Whatman No. 1 filter paper and ca 5.0 g of Na_2SO_4 into a 500-mL filtering flask. Extraction of sample is repeated by homogenization for 1 min with 100 mL of dichloromethane, and the sample is emptied and filtered onto the same Buchner funnel. The homogenizer flask is rinsed with ca 25 mL of dichloromethane, and the rinse is also passed through the funnel. The combined extracts are refiltered into a 500-mL round-bottom flask using a funnel and folded paper containing 2 g of anhydrous Na_2SO_4 to remove excess moisture. The filtering flask is rinsed with ca 20 mL of dichloromethane, and the rinsate is added to the funnel followed by washing the folded filter paper with ca 10 mL of dichloromethane. The combined extract is carefully concentrated to about 1–2 mL on a rotary evaporator with the water-bath at 30 °C, without allowing the flask to go dry. The concentrated extract is transferred to a 15-mL graduated centrifuge tube using a disposable pipet, and the round-bottom flask is rinsed with 1–2 mL of cyclohexane. The rinse is quantitatively transferred to the test-tube by washing the flask with small amounts of dichloromethane–cyclohexane (1 : 1), each time using a disposable pipet. The total volume in the test-tube should be exactly 7.5 mL. The resulting extract is filtered directly into a GPC system using a 10-mL loading syringe attached to a 0.45-μm filter. The sample is loaded on to the GPC system with a 5-mL loop. [GPC conditions: 60 × 2.5-cm i.d. chromatographic tube, ca 48-cm bed length, with 60 g of BioBeads SX-3 resin, 200–400 mesh; elution solvent dichloromethane–cyclohexane (1 : 1); and 5.0 mL min^{-1} flow rate]. Based on the results of the GPC calibration

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procedure, an appropriate volume of GPC eluate is dumped, and the sample residue fraction is collected in a 500-mL round-bottom flask. The solvent is evaporated to dryness on a rotary evaporator with a water-bath at 30 °C. The residue is quantitatively transferred with a disposable pipet to a 15-mL graduated test-tube by washing the flask with ca 10 mL of dichloromethane. The washings are concentrated to about 1 mL under a flow of nitrogen at 30 °C and diluted to 2 mL with dichloromethane. The residue solution (1-mL) is applied to a Bond-Elut aminopropyl cartridge (100-mg/1-mL) (Varian) conditioned with 1 mL of dichloromethane. The eluate is collected into a 15-mL graduated centrifuge tube immediately after application of the extract. Elution is completed by applying 3–5 mL of 1.5% methanol in dichloromethane. (Note: if recovery of aldicarb sulfoxide drops below 80% in two consecutive runs, the final wash volume of 1.5% methanol in dichloromethane should be carefully increased to obtain an 80–90% recovery of aldicarb sulfoxide.) The combined eluates are evaporated to dryness at 30 °C without overdrying using an N-evap. The residue is reconstituted in 200 μL of methanol, vortex mixed for 5 s (concentration 350 ng mL^{-1} of 10 μg kg^{-1} recovery), and filtered through a 0.45-μm fluoropolymer or nylon 66 membrane filter into a sample vial equipped with a glass insert for HPLC/fluorescence analysis.

(3) Milk. The recommended multiresidue method for oxime carbamates in milk, which is specific for aldicarb and its metabolites, is the method of Bennett *et al.*[23] The limit of detection (LOD) is 0.9 μg kg^{-1} for each analyte. Liquid whole milk (50 g) is weighed into a homogenizing vessel (1-pint glass jar with regular mouth or equivalent). A 200-mL portion of ethyl acetate–ethanol (95 : 9, v/v) solution and 125 g of anhydrous sodium sulfate are sequentially added to the vessel, and the sample mixture is homogenized [using an Omni Mixer (Omni International, Gainsville, VA, USA) or equivalent] for 2 min at medium speed. The extract is decanted into a 200-mL glass centrifuge bottle (heavy-wall, borosilicate glass bottle with screw-cap or equivalent), and the bottle is capped and centrifuged at 2000 rpm for 5 min. A 150-mL clear extract is pipetted into a 200-mL evaporation tube and concentrated at 40 °C using a Turbo Vap II (Zymark, Hopkinton, PA, USA) to a final volume of ca 2.0 mL. The concentrated extract is quantitatively transferred to a 15-mL graduated centrifuge tube by thoroughly rinsing the evaporation tube three times with 2 mL each of hexane. The extract is evaporated at 40 °C to a constant volume (ca 2 mL) with a gentle stream of nitrogen. Four Sep-Pak Plus C_{18} cartridges [(360-mg) (Waters) or equivalent] are stacked (1.44-g total C_{18}) with a 25-mL SPE reservoir on top and a 200-mL evaporation tube at the bottom. The cartridges are conditioned with 25 mL of acetonitrile by applying N_2 or air pressure through the SPE manifold until acetonitrile just covers the bottom of the reservoir. The cartridges are never allowed to go dry during and after conditioning. The concentrated extract, warmed to 40 °C, is partitioned with 2.0 mL of acetonitrile by agitating the mixture on a vortex mixer for 20–30 s. Once the phases have separated, the sample is frozen in an ice–water bath to immobilize the milk fats temporarily, and the acetonitrile extract is decanted into the reservoir of the C_{18} cartridges. Acetonitrile extraction and the freezing procedure are repeated four more times. (The sample freezing time decreases with each repetition; typical freezing times for a single step range from 7 to 2 min.) The combined extracts are eluted at a flow rate of 1–2 drops s^{-1} until liquid just covers the top cartridge. Elution is completed by

applying 14 mL of acetonitrile, after which vacuum is applied, until no more liquid flows through the cartridges. The eluate collected (ca 25 mL) is concentrated to a final volume of 1–1.5 mL at 40 °C on a Turbo Vap II. A Bond-Elut aminopropyl cartridge [(500-mg/6-mL)(Varian) or equivalent] is conditioned with 2 mL of dichloromethane–methanol (93 : 7, v/v) and never allowed to go dry after conditioning. The concentrated extract is applied to the aminopropyl cartridge and eluted by gravity flow into a 15-mL graduated glass centrifuge tube. The evaporation tube is thoroughly rinsed twice with 2 mL of dichloromethane–methanol (93 : 7, v/v), and the rinse is applied to the cartridge after every rinsing of the tube. The eluate is evaporated at 40 °C under a gentle stream of nitrogen to near dryness, the dry residue is reconstituted in methanol to a final volume of 1.0 mL, and the sample is filtered through a 0.45-μm PTFE membrane into an autosampler vial for HPLC/fluorescence analysis (37.5 g matrix mL^{-1}).

2.4 Soil

2.4.1 Nature of the residue

In general, pesticides degrade in the environment via the following processes: chemical (hydrolysis), biological, and photochemical. Chemical or biological degradation of oxime carbamates occurs through three principal routes: hydrolysis, oxidation, and conjugation.[40,41] In soil, the rate of degradation is influenced by the soil properties, temperature, the chemical stability of the oxime carbamate itself, and other factors. Based on toxicology considerations, the parent oxime carbamate (e.g., methomyl and oxamyl) or parent and metabolites (e.g., aldicarb and its metabolites) are monitored in soil.

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2.4.2 Rationale for methods

Oxime carbamates can be extracted from soil efficiently by Soxhlet extraction using organic solvents, such as acetonitrile–dichloromethane (1 : 1).[32] Another extraction technique involves mechanical shaking of the sample for 15 min or more in an aqueous solution of methanol[25] or in an acidified solution of methanol and acetonitrile. The resulting extract is purified by SPE employing an aminopropyl-bonded silica column or a C_{18} SPE cartridge, which are found to be effective in removing interfering matrix co-extractives from the analytes. Prior to chromatographic analysis, the purified extracts are passed through 0.2- or 0.45-μm syringe filters to remove colloidal co-extractives so as to minimize the degradation of the analytical column.

2.4.3 Description of method

The first recommended soil method for oxime carbamates is the method of Honing *et al.*[32] by HPLC/MS. The LOQ of the method, specifically for aldicarb, methomyl, and oxamyl, is 0.05 mg kg^{-1}. Soil (10 g) is Soxhlet extracted for 16 h with acetone–dichloromethane (1 : 1) using double-thickness cellulose extraction thimbles (80 × 22-mm i.d.). Prior to extraction, the Soxhlet system and the thimbles are cleaned for 14 h by refluxing with methanol. The extracts are removed and concentrated nearly to dryness in a rotary evaporator operating at 35 °C; evaporation to dryness

is then achieved under a nitrogen flow. The residue is dissolved in 500 μL of n-hexane and applied to the top of a 15-cm long glass column containing ca 2 g of aminopropyl-bonded silica (purified by Soxhlet extraction for 4 h) and eluted with 20 mL of dichloromethane–acetone (3 : 1). The aminopropyl-bonded silica column is protected against water by adding a 1-cm layer of anhydrous sodium sulfate on top of the sorbent. After collection, the eluate is evaporated nearly to dryness under a nitrogen flow, and the residue is reconstituted in 1 mL of water–methanol (4 : 1). The final extract should be filtered through a 0.22-μm PTFE syringe filter prior to HPLC/MS analysis.

An alternative method for aldicarb, aldicarb sulfone, and aldicarb sulfoxide in soil at an LOQ of 0.025 mg kg^{-1}, is the method of Johnson *et al.*[25] Soil (75 g) is weighed into a 500-mL Erlenmeyer flask and extracted with 150 mL of 4% methanol in deionized water using an orbital shaker for 2 h at approximately 250 rpm. The extract is decanted and centrifuged at 3600 rpm for 10 min. A graphite carbon cartridge (250-mg/3-mL) (Supelclean Envi-Carb, Supelco) is modified by placing a small plug of glass-fiber filter paper half-way down the barrel to filter out any suspended particulate matter. The cartridge is then conditioned successively with 5 mL of ethyl acetate, 15 mL of methanol, and 10 mL of a moderately hard water sample without allowing the cartridge to go dry. The extract is passed through the cartridge under vacuum at a rate of 7 mL min^{-1}. After the entire sample has passed through the cartridge, the flask is rinsed with ca 5 mL of moderately hard water, which is then added to the cartridge. The cartridge is eluted with 2 mL of methanol at a flow rate of approximately 5 mL min^{-1}. The sample is brought to a final volume of 2 mL with methanol, vortex mixed, and passed through a 0.45-μm Teflon syringe filter. The sample is placed in a 2-mL autosampler vial and stored at −20 °C until the time of the HPLC/fluorescence analysis.

Another alternative and fast method by HPLC/MS/MS, developed by DuPont Crop Protection, is specific for oxamyl and its oxime metabolite and may be applicable to other oxime carbamates and their metabolites. The LOQ of the method is 0.005 mg kg^{-1} for each analyte. A soil sample (20-g) is mixed with 20 mL of an extracting solution of 2.5% (v/v) formic acid in acetonitrile–methanol (3 : 1) in a 50-mL centrifuge bottle. The sample is heated in a 50 °C water-bath for 15 min, transferred to a mechanical shaker, and shaken for 15 min. The samples are stored overnight in a refrigerator to allow suspended soil particles to settle at the bottom of the tube or be centrifuged at 10 000 rpm for 20 min. A 0.5-mL aliquot is transferred into a graduated centrifuge tube and evaporated to dryness at 35 °C under a gentle stream of nitrogen. The sample is reconstituted in 1 mL of 0.1% formic acid in 10 mM ammonium acetate–methanol (9 : 1) with the help of a vortex mixer and ultrasonicator and filtered into an autosampler vial for analysis.

2.5 Water

2.5.1 Nature of the residue

Oxime carbamates are generally stable in aqueous solutions at pH 4–6. Their chemical degradation (hydrolysis) in water depends strongly on pH. Strongly basic conditions

promote fast hydrolysis of the methylcarbamoyl group to yield the noninsecticidal parent alcohol (an oxime, $R_1R_2C{=}N{-}OH$), amine, and other degradation products. Hydrolysis is accelerated at elevated temperature. Therefore, only the parent oxime carbamate (e.g., methomyl) or parent and metabolites that are insecticidally active and toxicologically relevant (e.g., thiodicarb and its metabolite methomyl; aldicarb and its sulfoxide and sulfone metabolites) are monitored in water.

The European drinking water guidelines[42] set a maximum admissible concentration of $0.10\,\mu g\,L^{-1}$ for individual pesticides and their related compounds in drinking water. The recommended methods for oxime carbamates and their metabolites in groundwater and surface water are suitable for detection limits below $0.10\,\mu g\,L^{-1}$. The first method is the HPLC/fluorescence multiresidue method of de Kok *et al.*[24] The second method is an HPLC/MS/MS method of DuPont Crop Protection which is specific for oxamyl and may be applicable for the analysis of other oxime carbamates in heavily polluted waters.

2.5.2 Rationale for method

To prevent degradation of oxime carbamates, water samples targeted for analysis must be preserved immediately after collection by acidification to pH 4–5 using a weak organic acid (e.g., glacial acetic acid). For samples with residual chlorine, sodium thiosulfate ($\sim$80 mg L^{-1}) should be added to the sample bottle prior to sample collection.[38] In addition, water samples should be stored at 4 °C and out of direct sunlight from the time of collection through analysis. Samples must be extracted and analyzed within 7 days after collection. Purification and concentration of oxime carbamates is by SPE using Bond-Elut C_{18}/OH-bonded silica cartridges (500 mg of adsorbent, 40-μm particle size) (Varian).[24] Alternative SPE cartridges that can be used for purification and concentration are stacked cartridges of Bond-Elut SAX (strong anion-exchange) and Oasis HLB. Filtration of the water samples prior to SPE is not required, provided that any particulates in the water are allowed to settle.

DropBooks

2.5.3 Description of method

The water sample (50-mL) is acidified to pH 3.0 by gradual addition of glacial acetic acid. A Bond-Elut C_{18}/OH SPE cartridge (500-mg/6-mL, 40-μm particle size) connected to a 75-mL reservoir (on top) is attached to a vacuum manifold and then sequentially conditioned with 2 mL of acetonitrile and 3 mL of water. The water sample is passed through the SPE cartridge at a flow rate of 2–5 mL min^{-1}, and the cartridge is washed with an extra 3 mL of water. When the water level just reaches the top of the column packing, 2 mL of acetonitrile are applied to elute the oxime carbamates into a calibrated centrifuge tube containing 10 μL of an internal standard solution (1 μg mL^{-1} landrin in dichloromethane). The extract is evaporated with nitrogen until ca 200 μL of residual water are left. The sample extract is made up to 1.0 mL with distilled water for HPLC/fluorescence analysis.

A sensitive and selective method, which is specific for oxamyl and may be applicable to other oxime carbamates and their metabolites in heavily polluted waters, involves the use of HPLC/MS/MS. A 75-mL reservoir is attached on top of a Bond-Elut SAX (1-g/6-mL) (Varian), which is connected to an Oasis HLB (1-g/20-mL)

(Waters). The stacked cartridges are conditioned with 25 mL of methanol followed by 25 mL of deionized water and are never allowed to go dry during and after conditioning. The water sample (100 mL) is then passed through the conditioned cartridges at a flow rate of 5–10 mL min^{-1}. The SAX cartridge acts as a filter for the water sample, and the analyte(s) is only retained at the Oasis HLB cartridge. The sample container is washed with 30 mL of water, and the wash is passed through the stacked cartridges. After removing the reservoir and SAX cartridge, the Oasis HLB cartridge is washed with 10 mL of water–methanol (7 : 3) and eluted with 12 mL of methanol–water (1 : 1) into a 15-mL glass centrifuge tube. The eluate is acidified with acetic acid (10 μL), evaporated at 30–35 °C under a nitrogen flow to reduce the volume to 7 mL, diluted with water to bring the final volume to 10 mL, and filtered using a 0.2-μm PTFE filter for analysis.

3 Conclusions and future directions

All the recommended residue methods for oxime carbamates generally produce mean recovery data in the range 80–110% with relative standard deviations below 20% at the stated LOQ and higher fortification levels. Furthermore, the recommended residue methods for crops and water are suitable for detection of oxime carbamates below 0.01 mg kg^{-1} and 0.10 μg L^{-1} levels, respectively, as mandated by the European Union (EU) New Baby Food Directive[43] and the EU Drinking Water Guidelines.[42] The recommended soil methods also satisfy the LOQ requirement of 0.05 mg kg^{-1} when the phytotoxic concentration in soil for sensitive crops or the toxic concentration for nontarget plant species is higher than 0.05 mg kg^{-1}.[42]

Currently, HPLC/fluorescence is still the most common technique for the determination of residues of oxime carbamates. With the introduction of ESI and APCI MS interfaces, HPLC/MS analysis for oxime carbamates in various sample matrices has become widespread. However, for a rapid, sensitive, and specific analysis of biological and environmental samples, HPLC/MS/MS is preferred to HPLC/MS and HPLC/fluorescence. With time, improved and affordable triple-quadrupole mass spectrometers will be available in more analytical laboratories. With stricter regulatory requirements, e.g., highly specific and conclusive methods with lower LOQ, HPLC/MS/MS will be a method of choice for oxime carbamates and their metabolites.

Methods for automated SPE cleanup and HPLC analysis with fluorescence, MS, or MS/MS detection have already been developed for the on-line monitoring of oxime carbamates and their polar metabolites in the aquatic environment[26–28,36] and also in the analysis of crop samples.[11] This involves the use of automated trace enrichment devices such as OSP2 (Merck, Darmstadt, Germany) and Prospekt (Spark, The Netherlands). On-line trace enrichment techniques use a precolumn containing an appropriate sorbent, which selectively retains the compounds of interest. For example, a water sample is enriched on an exchangeable cartridge, and, subsequently, the preconcentrated analytes are desorbed and transferred to the analytical column using a mobile phase gradient. Further advancement of the on-line trace enrichment technique for high-throughput analysis involves the use of a short column for rapid preconcentration of liquid samples and separation and detection of oxime carbamates and their metabolites by HPLC/MS/MS.

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References

1. C.D.S. Tomlin (ed.), *The Pesticide Manual*, twelfth edition, British Crop Protection Council, Farnham, Surrey (2000).
2. B.G. Schlagbauer and A.W. Schlagbauer, *Residue Rev.,* **42**, 1 (1972).
3. *Pesticide Analytical Manual*, Vol. II, Pesticide Regulations Section 180.269, US Food and Drug Administration, Washington, DC (1984).
4. R.F. Holt and H.L. Pease, *J. Agric. Food Chem.,* **24**, 263 (1976).
5. J.A. Itak, Y. Selisker, and D.P. Herzog, *Chemosphere,* **24**, 11 (1992).
6. E. Papadopoulou-Mourkidou and J. Patsias, *J. Chromatogr. A,* **726**, 99 (1996).
7. B.D. McGarvey, *J. Chromatogr. A,* **642**, 89 (1993).
8. 'Multi-residue Method 2: Pesticides Analysed with HPLC Procedures, Submethod 1: *N*-methylcarbamate Pesticides,' in "Analytical Methods for Pesticide Residues in Foodstuffs," sixth edition, General Inspectorate for Health Protection, Ministry of Public Health, Welfare and Sport, The Hague, pp. 1–10 (1996).
9. A. de Kok, M. Hiemstra, and C.P. Vreeker, *Chromatographia,* **24**, 469 (1987).
10. A. de Kok, M. Hiemstra, and C.P. Vreeker, *J. Chromatogr. A,* **507**, 459 (1990).
11. A. de Kok and M. Hiemstra, *J. AOAC Int.,* **75**, 1063 (1992).
12. J. Fillion, F. Sauve, and J. Selwyn, *J. AOAC Int.,* **83**, 698 (2000).
13. J. Cook, M.P. Beckett, W. Hammock, and M. Engel, *J. AOAC Int.,* **82**, 1420 (1999).
14. *Pesticide Analytical Manual*, Vol. I, Section 401, US Food and Drug Administration, Washington, DC (1994).
15. R.T. Krause, *J. Chromatogr.,* **185**, 615 (1979).
16. R.T. Krause, *J. Assoc. Off. Anal. Chem.*, **63**, 1114 (1980).
17. S.C. Stafford and W. Lin, *J. Agric Food Chem.,* **40**, 1026 (1992).
18. M. Okihashi, H. Obana, and S. Hori, *Analyst,* **123**, 711 (1998).
19. D. Chaput, *J. Assoc. Off. Anal. Chem.,* **71**, 542 (1988).
20. C. Russo, *Agric. Med.,* **122**, 313 (1992).
21. M.S. Ali, *J. Assoc. Off. Anal. Chem.,* **72**, 586 (1989).
22. M.S. Ali, J.D. White, R.S. Bakowski, N.K. Stapleton, K.A. Williams, R.C. Johnson, E.T. Phillippo, and R.W. Woods, *J. AOAC Int.,* **76**, 907 (1993).
23. D.A. Bennett, A.C. Chung, and S.M. Lee, *J. AOAC Int.,* **80**, 1065 (1997).
24. A. de Kok, M. Hiemstra, and U.A.Th. Brinkman, *J. Chromatogr. A,* **623**, 265 (1992).
25. K.A. Johnson, F.D. Harper, and C.P. Weisskopf, *J. Environ. Qual.,* **26**, 1435 (1997).
26. S. Chiron, A.F. Alba, and D. Barcelo, *Environ. Sci. Technol.,* **27**, 2352 (1993).
27. M. Hiemstra and A. de Kok, *J. Chromatogr. A,* **667**, 155 (1994).
28. J. Patsias and E. Papadopoulou-Mourkidou, *J. AOAC Int.,* **82**, 968 (1999).
29. T. Cairns, E.G. Siegmund, and G.M. Doose, *Biomed. Mass Spectrom.,* **10**, 24 (1983).
30. T.A. Bellar and W.L. Budde, *Anal. Chem.,* **60**, 2076 (1988).
31. C.H. Liu, G.C. Mattern, X. Yu, R.T. Rosen, and J.D. Rosen, *J. Agric. Food Chem.,* **39**, 718 (1991).
32. M. Honing, J. Riu, D. Barcelo, B.L.M. van Baar, and U.A.Th. Brinkman, *J. Chromatogr. A,* **733**, 283 (1996).

DeBooks

33. A. Di Corcia, C. Crescenzi, A. Lagana, and E. Sebastiani, *J. Agric. Food Chem.,* **44**, 1930 (1996).
34. M. Fernandez, Y. Pico, and J. Manes, *J. Chromatogr. A,* **871**, 43 (2000).
35. N. Wang and W.L. Budde, *Anal. Chem.,* **73**, 997 (2001).
36. J. Slobodnik, A.C. Hogenboom, J.J. Vreuls, J.A. Rontree, B.L.M. van Baar, W.M.A. Niessen, and U.A.Th. Brinkman, *J. Chromatogr. A,* **741**, 59 (1996).
37. L.Y.T. Li, D.A. Campbell, P.K. Bennett, and J. Henion, *Anal. Chem.,* **68**, 3397 (1996).
38. J.W. Munch (ed.), US EPA Method 531.3 Revision 3.1, US Environmental Protection Agency, Washington, DC (1995).
39. Code of Federal Regulations 40 – Protection of Environment, Parts 150–189, US Environmental Protection Agency, Washington, DC (2001).
40. B.D. Ripley and A.S.Y. Chau, 'Carbamate Pesticides,' in "Analysis of Pesticides in Water. Vol. III. Nitrogen-Containing Pesticides," ed. J.W. Robinson, A.S. Chau, and B.K. Afghan, CRC Press, Boca Raton, FL, pp. 1–182 (1982).
41. E.B. Tribaldo, 'Residue analysis of carbamate pesticides in water,' in "Food Science Technology," Marcel Dekker, New York, pp. 537–570 (2000).
42. European Commission, Directorate General Health and Consumer Protection, 'Guidance Document on Residue Analytical Methods,' SANCO/825/00 Rev. 6, European Commission, Brussels (2000).
43. Commission Directive 1999/39/EC, *Off. J. Eur. Commun.,* L 124, 8 (1999).

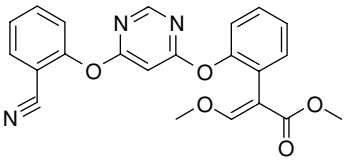
Individual compounds

DropBooks

Azoxystrobin

Materials to be analyzed	Avocado, barley, cabbage, carrot, grape, hops, leek, lentil, lettuce, melon, oil seed rape, onion, orange, pea, pear, plum, potato, strawberry, sugarbeet, tomato, wheat, soil, water, animal tissues, milk, egg and air
Instrumentation	High-performance liquid chromatography with triple-quadrupole mass spectrometry detection for crops, soil and water, gas chromatography with thermionic nitrogen-specific detection for animal products and gas chromatography with mass-selective detection (MSD) for air

1 Introduction

Chemical name (IUPAC)	Methyl (*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]-phenyl}-3-methoxyacrylate
Structural formula	
Empirical formula	$C_{22}H_{17}N_3O_5$
Molar mass	403.4
Melting point	116 °C
Vapor pressure	1.1×10^{-13} kPa (25 °C)
Solubility	In water (pH 7), 6 mg L^{-1} (20 °C). Low solubility in hexane, n-octanol; moderate solubility in methanol, toluene, acetone; high solubility in ethyl acetate, acetonitrile, dichloromethane
Stability	Stable to aqueous hydrolysis
Use pattern	Azoxystrobin is a synthetic analog of naturally occurring strobilurins and oudemansins. The preventative, curative, eradicant, translaminar and systemic properties facilitate control of a wide range of major plant pathogens in many crops

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Regulatory position	Azoxystrobin degrades rapidly and extensively in/on treated crops and in the environment. The residue definition of azoxystrobin in/on crops is for the parent compound only

2 Outline of methodology

2.1 Crop samples

Crop material is homogenized with acetonitrile–water (9 : 1, v/v). The crop extract is centrifuged and an aliquot is rotary evaporated to a small volume. The sample is subjected to a C_{18} solid-phase extraction (SPE) cleanup procedure. The concentrated eluate is subjected to liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis.

2.2 Soil

Soil is extracted twice with methanol–1 N hydrochloric acid (3 : 1, v/v), centrifuging between each extraction. An aliquot of the combined soil extract is diluted with acidified (pH 1) 5% (w/v) sodium chloride solution and subjected to liquid–liquid partitioning with dichloromethane. The dichloromethane extract is evaporated and the residue is dissolved in mobile phase prior to quantitation by LC/MS/MS.

DropBooks

2.3 Water

Water is acidified to pH 1 and passed through a C_{18} SPE cartridge on which azoxystrobin is retained. The column is dried under vacuum and azoxystrobin residue is eluted from the column. The eluate is adjusted to a known volume and an aliquot is analysed by LC/MS/MS.

2.4 Animal matrices

The sample is homogenized with acetonitrile. An aliquot of the extract is evaporated to dryness and the residual material is dissolved in ethyl acetate–toluene (3 : 1, v/v), and subjected to cleanup by gel permeation chromatography (GPC). After GPC, the sample is subjected to an alumina and Florisil SPE cleanup procedure. The concentrated eluate is analysed by gas chromatography/thermionic nitrogen-specific detection (GC/TSD).

2.5 Air

Air is sampled through a glass-fiber filter disk for 6 h at a rate of 2 L min^{-1}. The filter is placed in a vial containing acetonitrile and the vial is heated at 70 °C for 40 min. After cooling, the vial is ultrasonicated for 15 min. An aliquot of the acetonitrile is filtered and analysed by gas chromatography/mass spectrometry (GC/MS).

3 Apparatus

High-speed homogenizer
Laboratory mechanical shaker
Laboratory centrifuge
Polypropylene centrifuge bottles (250-mL)
Round-bottom flasks (100, 250-mL)
Measuring cylinder (100-mL)
Rotary vacuum evaporator, 40 °C bath temperature
Separatory funnels (100, 250-mL)
Vacuum manifold for SPE
C_{18} SPE columns (1-g/6-mL)
Silica SPE columns (200-mg/3-mL)
Alumina-N SPE columns (1-g/6-mL)
Florisil SPE columns (500-mg/3-mL)
Ultrasonic bath
Test-tubes (10-mL)
Filters disk (0.45-μm, 25-mm diameter)
Heating block for evaporation of samples
Glass-fiber filter disks and filter cassettes (25-mm-diameter)
Motorized air sampling units capable of 2 L min^{-1} flow
Glass screw-capped vials (22-mL)
Reversed-phase high-performance liquid chromatography (HPLC) column 50 mm × 3.2-mm i.d. with Kromasil 5-μm C_{18} packing
High-performance liquid chromatograph coupled to a triple-quadrupole mass spectrometer with an atmospheric pressure chemical ionization (APCI) source
Gel permeation chromatograph with a 60 mm × 25-mm i.d. column packed with Bio-Beads SX-3 (50-g)
Fused-silica capillary GC column, 15 m × 0.32-mm i.d., coated with trifluoropropylmethyl polysiloxane (0.5-μm film thickness)
Fused-silica capillary GC column, 30 m × 0.25-mm i.d., coated with 5% phenyl (equiv.) polysilphenylene–siloxane (0.25-μm film thickness)
Gas chromatograph fitted with a thermionic nitrogen-specific detector
Gas chromatograph fitted with a quadrupole mass-selective detector

4 Reagents

Acetonitrile, reagent grade
Dichloromethane, reagent grade

Ethyl acetate, reagent grade
Toluene, reagent grade
Acetone, reagent grade
Methanol, reagent grade
Hexane, reagent grade
Hydrochloric acid (0.1 N)
Acidified sodium chloride solution [5% (w/v) sodium chloride + 0.05 N hydrochloric acid]
Sodium chloride solution (5%, w/v)
Aqueous acetic acid solution (0.4%, v/v)
Sodium sulfate, anhydrous

5 Sampling and preparation

No specific sample preparation and processing are needed for this method.

6 Procedure

6.1 Extraction

For crop samples, homogenize 10 g of a prepared sample with acetonitrile–water [9 : 1 (v/v), 100 mL minus the water content of the sample] for 5 min and centrifuge the mixture at 3500 rpm for 5 min.

DropBooks

For soil samples, shake 20 g of a prepared air-dried soil sample with methanol–1 N hydrochloric acid [50 mL, 3 : 1 (v/v)] on a mechanical shaker for 30 min. Centrifuge the sample at 3500 rpm for 5 min and decant the supernatant into a round bottom flask (250-mL). Add a second 50 mL of methanol–1 N hydrochloric acid (3 : 1, v/v) to the soil sample and shake the mixture on a mechanical shaker for another 30 min. Centrifuge the sample at 3500 rpm for 5 min and then decant the supernatant into the same round-bottom flask (250-mL), combining the extracts.

For animal matrices, homogenize 10 g of a prepared sample with acetonitrile (50 mL) for 5 min and centrifuge the mixture at 3000 rpm for 3 min. Decant the supernatant extract into a measuring cylinder (100-mL) and adjust the volume of the extract to a known value.

For water, proceed to Section 6.2.3; for air, proceed to Section 6.2.5.

6.2 Sample cleanup procedures

6.2.1 Crops

Transfer an aliquot of the sample extract equivalent to 0.5-g of crop (5 mL) into a disposable test-tube (10-mL) and evaporate the sample in a heating block at 50 °C under a stream of dry air to <1 mL. Add 5 mL of acetonitrile–water (3 : 7, v/v) to the sample and ultrasonicate the solution to ensure that the sample is fully dissolved.

Precondition a C_{18} (EC) SPE column (1-g/6-mL) with methanol (5 mL) followed by another 5 mL of acetonitrile–water (3 : 7, v/v). Transfer the sample on to the column and allow it to percolate through the column under vacuum, discarding the column eluate. Wash the column with 5 mL of acetonitrile–water (3 : 7, v/v). Dry the column under high vacuum for 15 min and wash it with hexane (5 mL). Elute the azoxystrobin from the column with 5 mL of ethyl acetate–dichloromethane (11 : 9, v/v), and evaporate the eluate to dryness under a stream of air in a heating block at 50 °C. Dissolve the sample in 1 mL of acetonitrile–water (1 : 1, v/v) and filter the solution through a 0.45-μm syringe filter, transferring the filtrate to an autosampler vial ready for LC/MS/MS analysis.

For samples that contain a very high level of matrix co-extractives, e.g., hops, a secondary cleanup is required. Dissolve the evaporated C_{18} eluate in dichloromethane (2.5 mL). Precondition a silica SPE column (200-mg/3-mL) with dichloromethane (2.5 mL) and transfer the sample on to the column, discarding the column eluate. Wash the column with 2.5 mL of dichloromethane–ethyl acetate (19.5 : 0.5, v/v). Elute the azoxystrobin with 2.5 mL of dichloromethane–ethyl acetate (3 : 1, v/v). Evaporate the eluate to dryness in a heating block at 50 °C under a stream of clean, dry air and dissolve the residue in 1 mL of acetonitrile–water (1 : 1, v/v), transferring the solution to an autosampler vial ready for quantitation by LC/MS/MS.

6.2.2 Soil

Transfer an aliquot of the sample extract equivalent to 2 g of soil (10 mL) into a separatory funnel (100-mL) and add acidified sodium chloride solution (10 mL). Partition the sample with dichloromethane (2 × 10 mL), collecting the dichloromethane in a round-bottom flask (100-mL). Adjust the volume of the combined dichloromethane layers to 20 mL and remove an aliquot equivalent to 0.5-g of soil (5 mL). Evaporate the aliquot to dryness under a stream of dry air and dissolve the dry residue in 1 mL of acetonitrile–water (1 : 1, v/v), transferring the solution to an autosampler vial ready for quantitation by LC/MS/MS.

6.2.3 Water

Transfer an aliquot of the water sample (50 mL) into a round-bottom flask (100-mL) and add 0.5 mL of methanol and 1 mL of concentrated hydrochloric acid. Precondition a C_{18} (EC) SPE column (1-g/6-mL) with methanol (5 mL) followed by water (5 mL). Transfer the sample onto the column and draw it through the column under vacuum, discarding the column eluate. Wash the column with water (5 mL) and dry it under high vacuum for 15 min. Elute the azoxystrobin from the column with 2 × 2.5 mL of acetonitrile–water (7 : 3, v/v). Adjust the eluate to a final volume of 5 mL with acetonitrile–water (7 : 3, v/v) and transfer an aliquot of the sample to an autosampler vial ready for quantitiation by LC/MS/MS.

6.2.4 Animal matrices

Transfer an aliquot of the sample extract equivalent to 2 g of matrix into a round-bottom flask (250-mL). Add an equivalent volume of ethyl acetate to the sample to

prevent bumping and rotary evaporate the sample to dryness under reduced pressure with a water-bath temperature of 40 °C. Dissolve the residue in 4 mL of ethyl acetate–toluene (3 : 1, v/v) and transfer the solution to a suitable vial ready for GPC cleanup.

For milk, transfer the entire sample extract into a separatory funnel (250-mL), add an equivalent volume of dichloromethane plus a half equivalent volume of sodium chloride solution (5%, w/v). Shake the separatory funnel for 2 min and allow the phases to separate. Partially fill a glass filter funnel with anhydrous sodium sulfate (approximately 10 g) and filter the lower dichloromethane layer through the sodium sulfate, collecting the filtrate in a round-bottom flask (250-mL). Wash the sodium sulfate with dichloromethane (5 mL) and collect the washings in the same round-bottom flask. Rotary evaporate the sample to dryness under reduced pressure with a water-bath temperature of 40 °C. Dissolve the residue in 4 mL of ethyl acetate–toluene (3 : 1, v/v) and transfer the solution to a suitable vial ready for GPC cleanup.

Prior to use, each GPC column should be calibrated in order to establish the correct eluate collection time. In addition, the column should be equilibrated for at least 2 h prior to use.

GPC operating conditions

Column	600 mm × 25-mm i.d. glass column packed with Bio-Beads SX-3 (50 g)
Solvent system	Ethyl acetate–toluene (3 : 1, v/v)
Flow rate	5 mL min^{-1}
Injection volume	2 mL
Collection time	25–30 min

DropBooks

Place an alumina-N SPE column (1-g/6-mL) on top of a Florisil SPE column (500-mg/3-mL) using column connection adapters and precondition the columns with 20 mL of ethyl acetate–toluene (3 : 1, v/v). Transfer the collected fraction from the GPC cleanup onto the alumina-N column and allow it to percolate through both columns under gravity, collecting the eluate in a round-bottom flask (100-mL). Add 10 mL of ethyl acetate–toluene (3 : 1, v/v) to the alumina-N column and allow it to percolate through both columns under gravity, collecting the eluate in the same round-bottom flask (100-mL), combining the eluates. Rotary evaporate the sample to dryness under reduced pressure with a water-bath temperature of 40 °C. Dissolve the residue in 1 mL of toluene and transfer the solution to an autosampler vial ready for quantitation by gas chromatography (GC).

6.2.5 Air

Place a glass-fiber filter disk in a filter cassette, connect the cassette to a motorized air sampling pump and draw air through the cassette at a rate of 2 L min^{-1} for 6 h. After the sampling period, remove the filter disk and transfer it to a screw-capped glass vial (22-mL). Add 10 mL of acetonitrile to the vial and place it in a heating block set at 70 °C for 40 min. Allow the sample to cool and place it in an ultrasonic bath for 15 min. Filter an aliquot of the sample through a 0.45-μm syringe filter and transfer the filtrate to an autosampler vial ready for quantitation by GC.

6.3 Determination

6.3.1 Crops, soil and water

Inject an aliquot of the sample solution into the LC/MS/MS system. An HP1050 HPLC system coupled to a Perkin-Elmer API III triple-quadrupole mass spectrometer has been found to be suitable for this analysis.

Operating conditions

Column	KR100 5 C_{18} column, 50 mm × 3.2-mm i.d.
Column temperature	40 °C
Mobile phase	Aqueous acetonitrile (1 : 1, v/v) containing 0.4% (v/v) glacial acetic acid solution
Flow rate	0.8 mL min^{-1}
Injection volume	50 μL
Retention time	3.3 min
Ionization mode	APCI
Polarity	Positive
Nebulizer gas	Nitrogen at 60 psi
Auxiliary gas	Nitrogen at 1.8 L min^{-1}
Nebulizer temperature	480 °C
Collision gas	Argon–nitrogen (9 : 1, v/v)
Acquisition	Multiple reaction monitoring (MRM) monitoring of m/z 404 to 372 transition

6.3.2 Animal matrices

Inject an aliquot of the sample solution into the gas chromatograph. A Varian 3400 Series gas chromatograph fitted with a thermionic nitrogen-specific detector has been found to be suitable for this analysis.

Operating conditions

Column	Fused-silica capillary GC column, 15 m × 0.32-mm i.d., coated with trifluoropropylmethyl polysiloxane (0.5-μm film thickness)
Oven temperature	70 °C (held for 1.5 min), programmed at 30 °C min^{-1} to 220 °C and then at 10 °C min^{-1} to 300 °C (held for 10 min)
Injector	Septum-equipped programmable injector
Injector program	40 °C (held for 0.1 min), programmed at 180 °C min^{-1} to 250 °C (held for 25 min)
Injection volume	2 μL
Gas flow rates	Helium (carrier gas), 2.5 mL min^{-1} Helium (make-up gas), 27.5 mL min^{-1} Hydrogen, 4.5 mL min^{-1} Air, 175 mL min^{-1}
Detector	Temperature 300 °C, bead current 3.0–3.3 A

6.3.3 Air

Inject an aliquot of the sample solution into the gas chromatograph. An Agilent 6890 Series gas chromatograph fitted with a 5973 Series mass-selective detector has been found to be suitable for this analysis.

Operating conditions

Column	Fused silica capillary GC column 30 m × 0.25-mm i.d. coated with 5% phenyl (equiv.) polysilphenylene-siloxane (0.25-μm film thickness)
Oven temperature	100 °C (held for 1.0 min), programmed at 30 °C min^{-1} to 300 °C (held for 6 min)
Injector	Splitless, 250 °C
Injection volume	1 μL
Carrier gas flow rate	Helium at 1.0 mL min^{-1}, constant flow
Transfer line temperature	280 °C
Ionization mode	Electron impact
Detector calibration	Autotune
Acquisition type	Selected-ion monitoring
Acquisition masses	m/z 344, 388 and 403

7 Evaluation

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7.1 Method

Quantitation is performed by the single-point calibration technique. The calibration standard should be at a level similar to the expected residues and should be injected after every 3–4 samples throughout the sample batch. The mean response of the calibration standards which bracket the sample should be used for the residue calculation.

7.2 Recoveries, limit of detection and limit of quantification

7.2.1 Crops

The method has been validated in 22 different crop matrices at fortification levels from 0.01 to 0.5 mg kg^{-1}. Mean recoveries in each crop type ranged from 80 to 99% with the limit of detection (LOD) estimated to be 0.001–0.003 mg kg^{-1}.

7.2.2 Soil

The method has been validated in representative soil types at fortification levels from 0.02 to 1.0 mg kg^{-1}. Mean recoveries in each soil type ranged from 91 to 104% with the LOD estimated to be 0.002–0.004 mg kg^{-1}.

7.2.3 Water

The method has been validated in representative water types at fortification levels from 0.2 to 500 $\mu g\,L^{-1}$. Mean recoveries in each water type ranged from 77 to 93% with the LOD estimated to be 0.01–0.03 $\mu g\,L^{-1}$.

7.2.4 Animal matrices

The method has been validated in animal tissues and eggs at fortification levels from 0.01 to 0.1 $mg\,kg^{-1}$. Mean recoveries in each matrix ranged from 86 to 99% with the LOD estimated to be 0.002–0.004 $mg\,kg^{-1}$. The method has been validated in milk at fortification levels from 0.001 to 0.02 $mg\,kg^{-1}$. The mean recovery was 96% with the LOD estimated to be 0.0003–0.0005 $mg\,kg^{-1}$.

7.2.5 Air

The method has been validated at fortification levels of 2.16 and 21.6 μg of azoxystrobin adsorbed on the filter disks (equivalent to 0.003 and 0.03 $mg\,m^{-3}$ concentration in air, respectively). Mean recoveries ranged from 77 to 102% with the LOD estimated to be 0.005 μg (equivalent to 6.9 $ng\,m^{-3}$).

7.3 Calculation of residues

7.3.1 Crops, soil, water and animal matrices

The residue, expressed in $mg\,kg^{-1}$ ($\mu g\,L^{-1}$ for water), is calculated from the following equation:

$$\text{Residue} = \frac{\text{peak area (S)}}{\text{peak area (std)}} \times \frac{\text{standard conc.}}{\text{sample conc.}}$$

where

peak area (S) = peak response for sample
peak area (std) = mean peak response for bracketing standards
standard conc. = concentration of azoxystrobin standard ($\mu g\,mL^{-1}$)
sample conc. = final sample concentration ($g\,mL^{-1}$ for crop and soil and $mL\,mL^{-1}$ for water)

7.3.2 Air

The residue, expressed in micrograms adsorbed on the filter disks, is calculated from the following equation:

$$\text{Residue} = \frac{\text{peak area (S)}}{\text{peak area (std)}} \times \text{standard conc.}$$

where

peak area (S) = peak response for sample
peak area (std) = mean peak response for bracketing standards
standard conc. = concentration of azoxystrobin standard ($\mu g\ mL^{-1}$)

8 Important points

Sample extracts should not be stored in aqueous acetonitrile solution for more than 3 days before analysis by LC/MS/MS. Reduced recoveries of azoxystrobin have been observed in samples stored for longer periods of time.

Azoxystrobin residues in crops, soil and water are stable (>80%) during 2 years of frozen storage.

DFG method S19 (extended revision) multi-method L 00.00-34 of the Official Collection of Test Methods According to §35 LMBG[1] has been validated for azoxystrobin in orange, garlic, kohlrabi, camomile, fennel seed and tea. Extraction module E1, cleanup module GPC and detection module D4 (MSD) were used for orange, kohlrabi and garlic. Extraction module E2, cleanup modules GPC and C1 and detection module D4 (MSD) were used for camomile, fennel seed and tea.

Reference

1. Bundesinstitut für Gesundheitlichen Verbraucherschutz und Vetenärmedizin, 'Modular multi-method L 00.00-34 of the Official Collection of Test Methods According to §35 LMBH (Law of Food and Commodities),' Beuth Verlag, Berlin (1999).

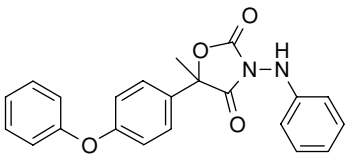
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Neil J. Robinson
Syngenta, Bracknell, UK

Famoxadone

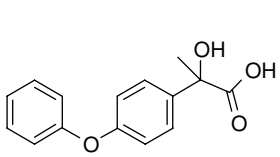
Materials to be analyzed	Grapes, wine, raisins, tomatoes, potatoes, cereals (grain and straw), cucurbits (cucumbers and melons), lettuce, peppers, soil, water
Instrumentation	Gas-chromatographic determination for plant materials; liquid chromatography/mass spectrometric determination for water and soil

1 Introduction

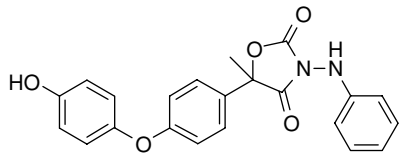
Chemical name (IUPAC)	3-Anilino-5-methyl-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione
Structural formula	
CAS Registry Number	131807-57-3
Empirical formula	$C_{22}H_{18}N_2O_4$
Molar mass	374.4
Melting point	141.3–142.3 °C
Vapor pressure	6.4×10^{-7} Pa at 20 °C
Solubility	Water: 52 μg L^{-1} at 20 °C Readily soluble in most organic solvents (except hexane)
Stability	Stable in acidic aqueous solution Unstable in neutral to basic conditions Stable in most organic solvents such as acetone, acetonitrile, and ethyl acetate
Other properties	The oxazolidinedione ring is unstable in aqueous basic solution; the N–N bond is also susceptible to cleavage when attacked by hydroxyl radicals
Use pattern	Famoxadone is an oxazolidinedione fungicide acknowledged for effective preventive effects and broad fungicidal spectrum. Famoxadone is used to control

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	a diverse array of foliar fungal diseases. Famoxadone works primarily by protective action on the plant surface
Regulatory position	The residue definition of famoxadone includes only famoxadone for plants and animals. For soil and water, IN-JS940 (CAS No. 157874-97-0) and IN-KZ007 have also been included in the residue definition.



IN-JS940



IN-KZ007

2 Outline of method

Plant materials are homogenized with a mixture of acetone and water, followed by ethyl acetate–cyclohexane partitioning. Soil is extracted with a mixture of methanol and sodium acetate buffer; for water, extraction is omitted. The solvent in the organic phase of the plant extract is removed by rotary evaporation, the residue is dissolved in ethyl acetate–cyclohexane, and the solution is subjected to a cleanup procedure using gel permeation chromatography (GPC) and silica gel column chromatography. For soil and water, the sample/extract is placed directly on a solid-phase extraction (SPE) cartridge for cleanup. The concentrated eluate is subjected to gas chromatography (GC) (for plant materials) or high-performance liquid chromatography (HPLC) (for soil and water samples).

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3 Apparatus

3.1 Plants

Coffee grinder
Mincer
Hand purée machine
Ultra Turrax homogenizer
Rotary evaporators, bath 40 °C
Evaporator, TurboVap LV
Ultrasonic bath
Graduated cylinder, 250-mL
Screw-top glass jar, 500-mL
Round-bottom flask, 500-mL
Glass funnels, 45- and 100-mm diameter
Graduated pipet, 10-mL
Round-bottom flask, long-necked, 15-mL

Tapered flasks, 25-mL
Graduated test-tubes with ground stoppers, 12–15-mL
Chromatographic column, 7-mm i.d., ca 23 cm long with a tapered outlet (ca 4-cm long and 1-mm i.d.)
Automated gel permeation chromatograph (CleanUp XL, Abimed Gilson) equipped with 5-mL loop and chromatographic tube, 25-mm i.d., 600-mm long, filled with 52-g of Bio-Beads, S-X3, 200–400 mesh, 33-cm gel bed length
GC Gas chromatograph equipped with a split/splitless injector, autosampler, DB-1 fused-silica column, 30 m × 0.25-mm i.d., 0.25-μm film thickness and electron capture detector
Gas chromatography/mass spectrometry (GC/MS) Gas chromatograph equipped with a split/splitless injector, autosampler, DB-5 MS fused-silica column, 15 m × 0.25-mm i.d., 0.25-μm film thickness and mass-selective detector

3.2 Soil and water

Mettler AE 163 and PM460 analytical balances
Benchtop centrifuge: IEC Model HN-SII; 6 × 50-mL fixed-angle solid rotor, 15-mL adapter sleeves
Beckman pH meter
Transfer pipets: Samco B/B PET transfer pipets
Pipettors: Pipetman, Gilson, manual, continuously adjustable volume, 100-, 200-, and 1000-μL and 10-mL
Pipet tips: Sorenson Multifit Research Pipet Tips: 5–200 and 100–1000 μL
Rainin Certified Disposable Pipet Tips: 10 mL
SPE cartridge (**do not substitute**): Mega Bond Elut, C_{18} bonded phase, 6-cc/1-g
SPE apparatus: Visiprep DL SPE manifold
Ultrasonicator: Bransonic ultrasonic cleaner, 0.75-gal (3.4 1-L) capacity
Vortex mixer
Graduated cylinders: Kimax brand, Red Stripe, glass, 100- and 500-mL and 1-L
HPLC sample vials: HP Amber with Teflon/silicone/Teflon septa, capacity 2-mL
Volumetric flasks, 200-mL
Centrifuge tubes: Becton-Dickinson BlueMax polypropylene, 15-mL
SPE column adapters: for 1-, 3- and 6-mL Bond Elut columns
SPE plastic reservoirs: 75-mL
Disposable flow control valve liners for SPE

Liquid chromatography/mass spectrometry (LC/MS) analysis
Quantitation system: Agilent Series 1100 liquid chromatograph
Chromsep Omnispher 3 C_{18} HPLC column, 100 × 4.6-mm i.d., 3-μm diameter particle size
Finnigan LCQ ion-trap mass spectrometer using ESI interface and Navigator 1.2 or XCalibur 1.0 SR1 software
Confirmatory system: Agilent Series 1100 liquid chromatograph
Eclipse XDB C_{18} HPLC column, 150 × 4.0-mm i.d., 3.5-μm particle size
MicroMass Quattro II triple-quadrupole mass spectrometer using an electrospray ionization (ESI) interface

4 Reagents

4.1 Plants

Acetone, cyclohexane, ethyl acetate, isooctane, n-hexane, toluene, pesticide grade
Eluent for GPC: cyclohexane–ethyl acetate (1 : 1, v/v)
Eluents for silica gel column chromatography: toluene (Eluent 2) and toluene–acetone (19 : 1, v/v) (Eluent 3)
Water, deionized
Sodium chloride, p.a. grade
Sodium sulfate, anhydrous heated at 550 °C for at least 2 h
Silica gel 60, Merck No. 7734
Quartz wool, extracted exhaustively with acetone
Filter paper, Type 595, ca 70-mm diameter extracted exhaustively with acetone, then folded

4.2 Soil and water

Phosphoric acid (H_3PO_4): 'Baker Analyzed' reagent, 85.0–87.0%
Formic acid (HCOOH): EM Suprapur, 98% min.
Glacial acetic acid (HOAc): EM OmniTrace, 99%
Acetonitrile (ACN), methanol (MeOH), water (H_2O): EM OmniSolv

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5 Sampling and preparation

Cucumbers and potato tubers are washed, then cut into portions (5-cm pieces and quarters, respectively), and frozen. Grapes are washed, stemmed, and frozen. Frozen pieces were homogenized using a cutter; portions (100-g for cucumbers and grapes, 50-g for potatoes) are weighed into the extraction vessels (screw-top jars) and stored at less than −18 °C until sample fortification and extraction.

For wine, 50-g portions are weighed into the extraction vessels just prior to fortification and extraction.

For raisins, 25-g portions are weighed into the extraction vessels (screw-top jars) and stored at less than −18 °C until sample fortification and extraction.

Grain is ground in a coffee grinder; 25-g portions are weighed into the extraction vessels just prior to fortification and extraction.

6 Procedure

6.1 Extraction

6.1.1 Plant material

Accurately weigh the appropriate portion (100-g for cucumber and grape, 50-g for potato and wine, 25-g for raisins and grain) into a 500-mL screw-top glass jar (or

beaker), and add the corresponding amount of water to adjust the final water content to 100 g (cucumber 4 mL, potato 62 mL, grape 12 mL, wine 50 mL, raisins 92 mL, wheat grain 96 mL). The dry masses of cucumber, potato, grape, and raisin are determined beforehand (dry for at least 12 h by using a drying cabinet at 105 °C). Add 200 mL of acetone (dispenser or graduated cylinder). Homogenize samples for 2 min using an Ultra Turrax (6000 rpm). Add 35 g of sodium chloride and 100 mL of ethyl acetate–cyclohexane (1 : 1, v/v). Homogenize the samples for 1 min by using an Ultra Turrax (6000 rpm). Allow the phases to separate (30–60 min). Measure out 200 mL (V_{R_1}) of the upper organic phase (graduated cylinder). Filter the organic phase through a glass funnel (the outlet is plugged with glass wool and covered with 100 g of anhydrous sodium sulfate), and collect the organic phase in a 500-mL round-bottom flask. Rinse the filter cake and graduated cylinder four times with ca 20 mL of ethyl acetate–cyclohexane (1 : 1, v/v). Combine the rinsates and filtered organic phase. Rotary evaporate the solution to generate an aqueous residue (1-mL, but not to dryness). Keep the water-bath at 40 °C. Add exactly 7.5 mL of ethyl acetate (pipet), and dissolve the residue from the glass surface by putting the flask in an ultrasonic bath (15 s). Add 5 g of anhydrous sodium sulfate–sodium chloride (1 : 1, w/w) to the flask contents, and swirl the flask. Add exactly 7.5 mL of cyclohexane (graduated 10-mL pipet) to obtain a total extract volume of 15 mL (V_{R_2}), and shake the sample vigorously. Allow the salt mixture to settle, and filter the extract through a glass funnel (diameter 45 mm) containing a folded filter. Collect the filtrate in a glass tube for GPC cleanup.

6.1.2 Soil

Add 20 mL of MeOH–30 mM NaOAc solution (4 : 1, v/v; NaOAc adjusted to pH 2.5–3) to the soil, and then the mixture should be vortex mixed for 30 s, sonicated for 5 min, vortex mixed momentarily, and centrifuged for 5 min at 2500 rpm. Decant the supernatant into a clean 100-mL graduated cylinder. Repeat the first two steps twice for a total of 60 mL of extraction solution used (dilute to 60 mL with MeOH as necessary). Transfer the solution into a glass, capped bottle (pour back and forth twice to mix).

6.1.3 Water

Water subsamples for analysis are prepared for cleanup by filling 200-mL volumetric flasks to volume and adding 10 mL of MeOH and 0.10 mL of concentrated phosphoric acid to adjust to approximately 5% MeOH and 0.01% H_3PO_4 by volume. All samples are stored at 4 ± 2 °C until cleanup.

6.2 Cleanup

6.2.1 Plants

GPC cleanup: Extracts obtained from Section 6.1.1 are purified using GPC with Bio-Beads S-X3.

GPC conditions

Flow rate	5 mL min^{-1}
Eluent	Ethyl acetate–cyclohexane (1 : 1, v/v)
Injection volume	5-mL (V_{R_3}), injection loop
Program	Dump: 17 min (85 mL)
	Collect: 22 min (110 mL)
	Wash: 0 min

Inject a 5-mL aliquot (V_{R_3}) of the sample extract via an injection loop. Collect the GPC fraction (110 mL) in an evaporation flask (150-mL round-bottom flask, long-necked). Rotary evaporate the sample carefully to 1 mL (but not to dryness) at 40 °C and 350–200 mbar (slow flask rotation, immerse flask slightly).

In order to obtain clean sample extracts for gas chromatography/electron capture detection (GC/ECD) detection, a supplementary cleanup on a small silica gel column is necessary. For preparation of silica gel columns: chromatographic column (the column must not run dry during the fractionation procedure): add in the following order – glass wool plug, 1.0 g of deactivated silica gel (1.5% water) and a 5–10-mm layer of anhydrous sodium sulfate. Pre-wash the column with 5 mL of hexane. For preparation and fractionation of sample extracts: add 10 mL of toluene to the residue obtained from GPC cleanup. Rotary evaporate the sample carefully to 1 mL (but not to dryness) at 40 °C and 150–50 mbar (slow flask rotation, immerse flask slightly). Add again 10 mL of toluene. Rotary evaporate the sample carefully to 1 mL (but not to dryness) at 40 °C and 350–200 mbar (slow flask rotation, immerse flask slightly). If the solution still has a residual odor of ethyl acetate, repeat the toluene steps. Transfer the toluene solution on to the silica gel column by using a Pasteur pipet. The initial eluate can be discarded. Place a 12–15-mL graduated test-tube that will accept a ground-glass stopper under the column (a 25-mL pear-shaped evaporation flask may be substituted). Pipet 2.0 mL of Eluent 2 (100% toluene) into the round-bottom flask described in Section 6.1.1. Swirl the flask, and dissolve the residues from the glass surface by using a ultrasonic bath. Transfer the toluene solution onto the silica gel column, and collect this eluate as E2. Add an additional 6 mL of Eluent 2 to the silica gel column. Add the column eluate to E2. Discard/store Eluate E2. Exchange the graduated test-tube under the column. Pipet 2.0 mL of Eluent 3 (toluene–acetone, 19 : 1, v/v) into the round-bottom flask to rinse it. Transfer this solution onto the silica gel column and collect this eluate as E3. Add an additional 6 mL of Eluent 3 to the silica gel column. Add the column eluate to E3. For potato, wine, and raisin, Eluate E3 is concentrated to 1–2 mL by using a Zymark evaporator TurboVap LV. Add toluene to adjust the final volumes (V_{END}) of 2.5 mL for potato and wine and 5 mL for raisin. For cucumber and grape, the final volumes (V_{END}) are adjusted to 10 mL with toluene.

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6.2.2 Soil

For each sample, condition C_{18} SPE (6-cc/1-g) cartridges with 6 mL of methanol and 3 × 6 mL of 0.005% aqueous H_3PO_4 in 10% MeOH–H_2O. *Stop Flow* as the solution reaches the top of the sorbent (the SPE sorbent must not be allowed to go

dry through the first five steps). Add 4 mL of 0.005% H_3PO_4 in 10% MeOH–H_2O to each SPE cartridge, and install a 75-mL reservoir equipped with stop valve over the SPE cartridge. Pipet 6 mL of extract into the reservoir and dilute the sample with 44 mL of 0.005% aqueous H_3PO_4. *Apply Vacuum*, and allow the sample to flow through the SPE cartridge at a fast drip (5–10 mL min^{-1}). When the reservoir is just empty, *Stop Flow*. Repeat the second and third steps. Close the stop valve and remove the reservoir with stop valve attached. Add 5 mL of MeOH to each reservoir, vortex mix the contents gently, dilute the sample with 45 mL of 0.005% aqueous H_3PO_4, and replace the reservoir on top of the SPE cartridge; *Resume Flow* through the SPE cartridge. As soon as the reservoir empties, *Stop Flow*, remove items above the SPE cartridge (reservoir, stopcock, adapter), and fill the SPE cartridge headspace with 0.005% aqueous H_3PO_4 so that the SPE cartridge inner walls will be relatively free of liquid drops when eluted. Continue full vacuum for 30 min after the solution passes through the SPE cartridge to dry the sorbent. *Break Vacuum* and install 15-mL centrifuge tubes under each SPE cartridge. Add 6 mL of 0.01% HCOOH in MeOH to each SPE cartridge, apply gentle vacuum to the cartridge, and collect the solution at a slow drip rate. Repeat the previous step with 4 mL of 0.01% HCOOH in MeOH. *Break Vacuum* when dripping stops. Recover the collection tubes, dilute the sample to 10 mL with MeOH; if necessary, vortex mix and centrifuge the sample for 5–10 min (2500 rpm). For all samples, add 0.8 mL of 0.01% HCOOH in MeOH–H_2O (3 : 8, v/v) to the autosampler vials labeled for samples, transfer 0.20 mL of the final extract to the respective autosampler vials, cap the vials, and vortex mix the samples prior to LC/MS analysis [use a 1 : 4 (v/v) dilution of the final extract]. For control and limit of quantification (LOQ) fortified samples, add 0.6 mL of aqueous 0.01% HCOOH to autosampler vials labeled for samples, transfer 0.6 mL of the final extract to the respective autosampler vials, cap the vials, and vortex mix the samples prior to LC/MS analysis [use a 1 : 1 (v/v) dilution of the final extract].

6.2.3 Water

Condition C_{18} SPE (6-cc/1-g) cartridges with 6 mL of methanol and 3 × 6 mL of 0.005% aqueous H_3PO_4. *Stop Flow* as the solution reaches the top of the sorbent (the SPE sorbent must not be allowed to go dry through the first five steps). Add 4 ml of 0.005% aqueous H_3PO_4 to the SPE headspace, *Start Flow*, and begin addition of sample. [Adjust the vacuum to produce a fast drip rate (5–10 mL min^{-1}), 30 min for a 200-mL application or 6.7 mL min^{-1}.] When the flasks are empty, *Stop Flow*, add 5 mL of MeOH to flasks, and swirl and rotate the flasks to rinse the sides. Pour MeOH into a reservoir, and gently vortex mix the sample. Add 45–50 mL of 0.005% aqueous H_3PO_4 to the flask, swirl and rotate the flask to rinse the sides, and add the flask contents to the reservoir to dilute the MeOH solution to approximately 10% (v/v). *Resume Flow* through the SPE cartridge. As soon as the reservoir empties, *Stop Flow*, remove items above the SPE cartridge (reservoir, stopcock, adapter), and fill the SPE cartridge headspace with 0.005% aqueous H_3PO_4 so that the SPE cartridge inner walls will be relatively free of liquid drops when eluted. Continue full vacuum for 30 min after the solution passes through the SPE cartridge to dry the sorbent. *Break Vacuum*, place 15-mL centrifuge tubes under the SPE cartridges. Add 6 mL of 0.01% HCOOH in MeOH to the headspace of the respective SPE cartridges. Open the stopcock under

the SPE cartridge and allow solvent to flow at a slow drip rate through the cartridge into the collection tube (a gentle vacuum may be applied). Repeat step 6 with 4 mL of 0.005% aqueous H_3PO_4, and continue the collection of acidified MeOH in tubes; use the vacuum to collect the total solution. Recover the collection tubes; if necessary, the samples should be diluted to 10 mL with MeOH, vortex mixed, and centrifuged for 5–10 min (2500 rpm). For all samples, add 0.8 mL of 0.01% HCOOH in MeOH–H_2O (3 : 8, v/v) to autosampler vials labeled for samples, transfer 0.20 mL of the final extract into respective autosampler vials, cap the vials, and vortex mix the samples prior to LC/MS analysis [use a 1 : 4 (v/v) dilution of the final extract]. For control and LOQ fortified samples, add 0.6 mL of aqueous 0.01% HCOOH to autosampler vials labeled for samples, transfer 0.6 mL of the final extract into the respective autosampler vials, cap the vials, and vortex mix the samples prior to LC/MS analysis [use a 1:1 (v/v) dilution of the final extract].

6.3 Determination

6.3.1 Plant material

Inject an aliquot of the GC-ready sample solution into the gas chromatograph.

For cucumber, potato, grape, wine, raisin, and grain extracts

Gas chromatograph	Hewlett-Packard Model 6890 with electron capture detector
Software	Hewlett-Packard GC ChemStation Rev. A.05.04
Column	30-m fused-silica capillary column, DB-1 (J&W Scientific), 0.25-mm i.d., film thickness 0.25-μm
Carrier gas flow rate	Argon-methane (19 : 1, v/v), 1.1 mL min^{-1}
Temperatures	
Oven	Initial temperature 100 °C, held for 2 min, increased at 7 °C min^{-1} to 250 °C, held for 6 min, increased at 20 °C min^{-1} to 290 °C, held for 17 min
Detector	300 °C
Injector	250 °C
Injection volume	2-μL split injection, split flow 27.1 mL min^{-1} (split ratio 1 : 25) (Autosampler HP 7673 Series)
Inlet glass liner	4-mm i.d. split liner with cup (e.g., HP Part No. 18740-80190) packed with a 5-mm layer of 3% OV-101 on GasChrom Q, 100–120 mesh (WGA, Düsseldorf, Germany)
Retention time	Famoxadone, 36.6 min

DropBooks

6.3.2 Soil and water

LC/MS systems. Agilent 1100 Series HPLC system connected to a Thermoquest-Finnigan LCQ ion-trap mass spectrometer using an ESI interface at atmospheric pressure.

HPLC components: G1322A vacuum degasser, G1312A binary pump, G1316A column compartment, and G1313A autosampler. Instrument operation and data acquisition are controlled using XCalibur 1.0 SR1 software. The LCQ is operated in the selected ion monitoring (SIM) negative ion mode for quantitative analysis.

Agilent 1100 Series HPLC system connected to a MicroMass Quattro II triple-quadrupole mass spectrometer using an ESI interface at atmospheric pressure. HPLC components: G1322A vacuum degasser, G1312A binary pump, G1316A column compartment, and G1329A refrigerated autosampler. Data acquisition and system control: MassLynx version 3.1 software. The Quattro II is operated in the LC/MS/MS negative ion mode with selected reaction monitoring (SRM) detector output for confirmatory analysis.

Operating conditions

A. HPLC operating conditions for quantitative analysis on the LCQ ion-trap mass spectrometer

Injection volume	100.0 μL
Column	Chromsep Omnispher 3 C_{18} HPLC column, 100 × 4.6-mm i.d., 3-μm particle size
Column temperature	29 °C
Solvent A	0.005 M HOAc in distilled, deionized water
Solvent B	0.005 M HOAc in ACN
Post run time	4.0 min (re-equilibration to initial conditions)

Program

Time	Flow rate (mL min^{-1})	A (%)	B (%)	Comments
0.0	0.8	95	5	
3.0	0.8	95	5	
8.0	0.8	35	65	
16.0	0.47	16.3	83.7	
16.1	0.8	16.3	83.7	
20.0	0.8	1	99	End run (post run +4 min)

Approximate analyte retention times	IN-JS940 10.2 min, IN-KZ007 11.4 min, famoxadone 14.8 min

B. HPLC operating conditions for confirmatory analysis on the Quattro II triple-quadrupole mass spectrometer

Injection volume	100.0 μL
Column	Eclipse XDB C_{18} HPLC column, 150 × 4.6-mm i.d., 3.5-μm particle size
Column temperature	40 °C
Solvent A	0.05% HOAc in distilled, deionized water
Solvent B	0.05% HOAc in MeOH

Program

Time	Flow rate (mL min^{-1})	A (%)	B (%)	Comments
0.0	0.8	95	5	
3.0	0.8	95	5	
8.0	0.8	35	65	
16.0	0.47	16.3	83.7	
20.0	0.8	1	99	
20.4	0.8	95	5	
24	0.8	95	5	End run

Approximate analyte retention times IN-JS940 14.1 min, IN-KZ007 14.2 min, famoxadone 17.4 min

C. MS operating conditions for quantitative analysis on the LCQ ion-trap mass spectrometer

Interface ESI
Mode Negative ion SIM
Sheath gas flow 60 psi
Auxiliary gas 15 psi
Heated capillary temperature 150 °C
Source voltage 4.5 kV
Divert valve 0.0–7.0 min to waste, 7.0–16.8 min to source, 16.8–20.0 min to waste
MS detector settings
Segment 1 0–11 min, SIM m/z 257 ± 0.5
Segment 2 11–14 min, SIM m/z 389 ± 0.5
Segment 3 14–17 min, SIM m/z 373 ± 0.5
MS run time 17 min

D. MS/MS operating conditions for confirmatory analysis on the Quattro II triple-quadrupole mass spectrometer

Interface ESI
Mode Negative ion MS/MS-MRM
Divert valve 0–12.0 min to waste, 12.0–18.5 min to source, 18.5–24.0 min to waste
Capillary 4.00 kV
HV lens 0.90 kV
Cone 35 V
Skimmer offset 2 V
RF lens 0.0 V
Source temperature 100 °C

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MS1		*MS2*	
Ion energy	2.0 V	*Ion energy*	2.0 V
Ion energy ramp	0.0 V	*Ion energy ramp*	0.0 V
LM resolution	8.0 V	*LM resolution*	6.0 V
HM resolution	8.0 V	*HM resolution*	6.0 V
Lens 5	100 V	*Lens 7*	250 V
Lens 6	5 V	*Lens 8*	157 V
Multiplier 1	700 V	*Lens 9*	2 V
		Multiplier 1	700 V

Pressures
Analyzer vacuum 3.45×10^{-5} mbar
Gas cell 2.0×10^{-3} mbar

Function 1 (IN-JS940)
Retention window 12.450–16.000 min

Reaction	Dwell (s)	Cone voltage (V)	Col. energy
1: 256.90 > 212.80	0.40	35.0	11.0

Function 2 (IN-KZ007)
Retention window 12.500–16.000 min

Reaction	Dwell (s)	Cone voltage (V)	Col. energy
1: 388.90 > 344.70	0.40	40.0	12.0

Function 3 (famoxadone)
Retention window 16.000–18.500 min

Reaction	Dwell (s)	Cone voltage (V)	Col. energy
1: 372.90 > 281.90	0.50	45.0	19.0

7 Evaluation

7.1 Method

7.1.1 Plant material

Standard solutions, containing famoxadone, are injected before every two sample extracts in each gas-chromatographic sequence. The concentration of analyte found expressed in $\mu g\,mL^{-1}$ is calculated using the peak height (GC/ECD) or peak area (GC/MS) in integrator units (IU = counts) obtained from the standard solution injected close to the sample extract during the gas-chromatographic sequence. Analytical standard levels of famoxadone are prepared at levels that are approximately equal to

those expected in the fortified samples. Single-point calibration, after two injected sample extracts, is done to compensate for a possible decrease in detection sensitivity, when injecting a large number of sample extracts. An approach using multiple external standard levels and linear regression calculations would also be appropriate. Other approaches to calibration and calculation might also work but must be evaluated prior to use.

7.1.2 Soil and water

Famoxadone, IN-JS940, and IN-KZ007 residues are measured in soil ($\mu g\,kg^{-1}$), sediment ($\mu g\,kg^{-1}$), and water ($\mu g\,L^{-1}$). Quantification is based on analyte response in calibration standards and sample extract analyses determined as $\mu g\,mL^{-1}$. Calibration standard runs are analyzed before and after every 1–4 samples in each analytical set. Analyte quantification is based on (1) linear regression analysis of (y-axis) analyte concentration ($\mu g\,mL^{-1}$) and (x-axis) analyte peak area response or (2) the average response factor determined from the appropriate calibration standards. The SLOPE and INTERCEPT functions of Microsoft Excel are used to determine slope and intercept. The AVERAGE and STDEV functions of Microsoft Excel are used to determine average response factors and standard deviations.

7.2 Recoveries, limit of detection, and limit of quantification

7.2.1 Plant material

With fortification levels between 0.010 and 0.50 $mg\,kg^{-1}$, average recoveries from untreated plant matrices range from 78 to 106% with the limit of quantification (LOQ) and the corresponding limit of detection (LOD) of famoxadone being as follows:

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Validated matrix	LOQ ($mg\,kg^{-1}$)	LOD ($mg\,kg^{-1}$)
Cucumber	0.020	0.01
Potato	0.010	0.005
Grain	0.010	0.005
Grapes	0.020	0.01
Wine	0.010	0.005
Raisins	0.050	0.02

7.2.2 Soil

With fortification levels between 0.010 and 0.10 $mg\,kg^{-1}$, average recoveries of famoxadone from untreated soil range from 86 to 110% with an LOQ of 0.010 $mg\,kg^{-1}$ and the corresponding LOD being 0.003 $mg\,kg^{-1}$.

7.2.3 Water

With fortification levels between 0.10 and 1.0 $\mu g\,kg^{-1}$, average recoveries of famoxadone from untreated water range from 79 to 107% with an LOQ of 0.10 $\mu g\,kg^{-1}$ and the corresponding LOD being 0.03 $\mu g\,kg^{-1}$.

7.3 Calculation of residues

7.3.1 Plants

The residues (R) of famoxadone in all plant samples, expressed in mg kg^{-1}, are calculated using an external standard with one-point calibration according to the following equations:

$$R = \left(P_A \times C_{St} \times V_{End} \times V_{Ex} \times V_{R_2}\right) \Big/ \left(P_{St} \times V_{R_1} \times V_{R_3} \times W\right) \quad (1)$$

where

P_A = peak height of analytes in the final sample solution measured by GC/ECD, in counts
P_{St} = peak height of analytes in the standard solution measured by GC/ECD, in counts
C_{St} = concentration of the standard solution, in μg mL^{-1}
W = weight of the analytical sample (25 g for raisin, 50 g for potato and wine or 100 g for cucumber and grape)
V_{Ex} = empirical volume of organic extract (285 mL) (see *Note*)
V_{R_1} = volume of V_{Ex} used for cleanup (200 mL)
V_{R_2} = volume of the solution before cleanup by GPC (15 mL)
V_{R_3} = aliquot of V_{R_2} injected for GPC (5 mL)
V_{End} = volume of the sample solution after cleanup by GPC and mini-silica gel SC (2.5 mL for grain, potato and wine, and 10 mL for cucumber and grape)

Note: Empirical volume of organic extract (V_{Ex}): acetone (200 mL) + ethyl acetate–cyclohexane [1 : 1 (v/v), 100 mL] − empirical volume shrinkage (5 mL) − empirical transfer of acetone into the aqueous phase during the liquid–liquid partition (10 mL) = V_{Ex} = 285 mL.

7.3.2 Soil and water

Calculation to determine μg kg^{-1} of famoxadone, IN-KZ007, and IN-JS940 found in soil and sediment test samples by linear regression analysis:

$$\mu g\,kg^{-1}\ \text{found} = \frac{(\text{slope} \times \text{PA} + \text{intercept})(\text{DF})(\text{FV/AV})(\text{FX})}{\text{SW}} \quad (2)$$

Calculation to determine μg L^{-1} found in water test samples by linear regression analysis:

$$\mu g\,L^{-1}\ \text{found} = \frac{(\text{slope} \times \text{PA} + \text{intercept})(\text{FV/AV})(\text{FX})}{\text{SV}} \quad (3)$$

Calculation to determine μg kg^{-1} found in soil and sediment test samples by average response factor analysis:

$$\mu g\,kg^{-1}\ \text{found} = \frac{(\text{analyte peak area}/\text{RF}_{\text{avg.}})(\text{DF})(\text{FV/AV})(\text{FX})}{\text{SW}} \quad (4)$$

Calculation to determine ppb ($\mu g\,L^{-1}$) found in water test samples by average response factor analysis:

$$\mu g\,L^{-1}\ \text{found} = \frac{(\text{analyte peak area}/RF_{avg.})(FV/AV)(FX)}{SV} \quad (5)$$

where

PA = analyte peak area
DF = dilution factor (60 mL extract/12 mL aliquot = 5) used for soil and sediment samples
FV = final extract volume (1.0 or 1.2 mL) of the pre-analysis diluted sample
AV = aliquot volume of final extract used in pre-analysis dilution (0.2 or 0.6 mL)
FX = final extract volume (10.0 mL)
SW = sample weight (0.010 kg) of soil or sediment extracted
SV = sample volume (0.20 L) of water extracted
$RF_{avg.}$ = average response factor [peak area/($\mu g\,mL^{-1}$)] for analyte

The percentage recovery for soil, sediment, and water in fortified samples is determined as follows:

$$\text{Recovery}(\%) = (\mu g\,kg^{-1}\text{found}) \times 100/(\mu g\,kg^{-1}\text{applied}) \quad (6)$$

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8 Important points

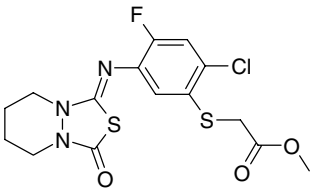
Famoxadone residues in most crops are stable during 12 months of frozen storage.

Kathryn M. Jernberg
DuPont Crop Protection, Newark, DE, USA

Fluthiacet-methyl

Materials to be analyzed	Corn, green corn (silage), soil, water
Instrumentation	Gas-chromatographic determination, high-performance liquid chromatographic determination

1 Introduction

Chemical name (IUPAC)	Methyl [2-chloro-4-fluoro-5-(5,6,7,8-tetrahydro-3-oxo-1*H*,3*H*-[1,3,4]thiadiazolo[3,4-*a*]pyridazin-1-ylidene-amino)phenylthio]acetate
Structural formula	
Empirical formula	$C_{15}H_{15}ClFN_3O_3S_2$
Molar mass	403.9
Melting point	105.0–106.5 °C
Vapor pressure	4.41×10^{-4} mPa (25 °C)
Solubility	Water 0.85 (distilled), 0.78 (pH 5 and 7), 0.22 (pH 9) mg L^{-1}(25 °C); methanol 4.41, acetone 101, toluene 84, n-octanol 1.86, acetonitrile 68.7, ethyl acetate 73.5, dichloromethane 9 g L^{-1} (25 °C), n-hexane 0.232 g L^{-1} (20 °C)
Stability	DT_{50} in water, 485 days (pH 5), 18 days (pH 7), 0.2 days (pH 9)
Use pattern	Fluthiacet-methyl is a selective contact herbicide for the post-emergence control of broad-leaved weeds in corn and soybeans.
Regulatory position	The residue definition is for the parent, fluthiacet-methyl, only.

Handbook of Residue Analytical Methods for Agrochemicals.
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2 Outline of method

Fluthiacet-methyl in crops is homogenized in an aqueous methanol solvent mixture; the methanol is evaporated and fluthiacet-methyl is extracted with n-hexane. Fluthiacet-methyl is partitioned into acetonitrile, purified with a solid-phase extraction (SPE) cartridge and then quantified by gas chromatography/flame thermionic detection (GC/FTD). Fluthiacet-methyl in soil is extracted with acetonitrile by sonication after shaking. Fluthiacet-methyl is partitioned into n-hexane, purified with an SPE cartridge and quantified by high-performance liquid chromatography (HPLC). Fluthiacet-methyl in water is extracted with dichloromethane and a concentrate of the dichloromethane extract is purified by silica gel column chromatography for gas chromatography/electron capture detection (GC/ECD) analysis.

3 Equipment

Ultracentrifugal mill
Homogenizer: Polytron mixer
Mechanical shaker
Ultrasonic cleaner: UC-6100, 600 W, 28 kHz(Sharp)
Round-bottom flasks: 300-mL, 200-mL and 1-L with ground joints
Pear-shaped flasks: 50- and 300-mL
Conical beaker: 300-mL
Buchner funnel: 10-cm i.d.
Separatory funnels: 1000-, 300-, and 200-mL
Glass funnel
Glass chromatography column: 400 × 15-mm i.d. with a stopcock
Column preparation: A silica gel column is prepared by packing a slurry of silica gel (10 g) in n-hexane–acetone (9 : 1, v/v) into a glass chromatography column. About a 1-cm layer of anhydrous sodium sulfate is placed above and below the silica gel bed.
SPE cartridge column:
  Bond Elut LRC SI (Varian) Conditioning with 10 mL of n-hexane–ethyl acetate (4 : 1, v/v): Sep-Pak Plus NH_2 (Waters)
  Conditioning with 10 mL of n-hexane-ethyl acetate (4 : 1 v/v): Supelclean Envi-Carb (500 mg) + LC-NH_2 (500 mg) (Supelco)
  Conditioning with 10 mL of acetonitrile–toluene (3 : 1, v/v)
Rotary vacuum evaporator, 50 °C bath temperature

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4 Reagents

Acetone, acetonitrile, dichloromethane, ethyl acetate, n-hexane, methanol and toluene: pesticide residue analysis grade
Distilled water: HPLC grade
Sodium chloride: special grade

Anhydrous sodium sulfate: special grade
Silica gel: Wakogel C-20 (Wako Pure Chemical Industries, Ltd)
Fluthiacet-methyl: analytical standard material (K-I Chemical Research Institute Co., Ltd)
Fluthiacet-methyl standard solutions:
for GC/FTD: 0.05, 0.1, 0.4, 0.75 and 1.0 μg mL^{-1} in acetone
for GC/ECD: 0.01, 0.03, 0.05 and 0.125 μg mL^{-1} in acetone–n-hexane (1 : 9, v/v)
for HPLC: 0.03, 0.15, 0.3, 0.45 and 0.6 μg mL^{-1} in acetonitrile–water (7 : 3, v/v)

5 Sample preparation

Corn grains are ground with a mill. Green corn silage is cut into pieces of 1- or 2-cm size, ground and homogenized with a mill.

6 Procedure

6.1 Extraction

6.1.1 Plant material

For corn grains, weigh 5 g of the ground sample into a 300-mL conical beaker, add 100 mL of methanol–water (2 : 1, v/v) and homogenize the sample for 3 min with a Polytron homogenizer. Filter the homogenized sample through a filter paper on a Buchner funnel by suction and collect the filtrate in a 500-mL round-bottom flask. Wash the beaker and the residue with 70 mL of the same solvent mixture and filter them in a similar manner. Combine the filtrate and concentrate it at 50 °C or lower under reduced pressure to 50 mL. For green corn, weigh 10 g of the ground sample into a 200-mL round-bottom flask, add 50 mL of methanol–water (2 : 1, v/v) and shake the flask for 30 min. Filter the extract by suction and wash the flask and the residue with 30 mL of the mixed solvent. Combine and concentrate the extract and washings to 20 mL at 50 °C or lower under reduced pressure.

6.1.2 Soil

Weigh 30 g (dry soil base) of soil into a 300-mL round-bottom flask, add 90 mL of acetonitrile and 30 mL of water, shake the mixture for 10 min and sonicate it for 30 min. Filter the mixture through a filter paper on a Buchner funnel by suction and collect the filtrate in a 500-mL round-bottom flask. Wash the beaker and the residue with 60 mL of acetone and filter the washings. Combine and concentrate the filtrates to 20 mL at 50 °C or lower under reduced pressure.

6.1.3 Water

Place 400 mL of groundwater sample in a 1-L separatory funnel, and extract the aqueous phase twice with 200 mL of dichloromethane.

6.2 Cleanup

6.2.1 Plant material

Transfer the concentrate from Section 6.1.1 into a 200-mL separatory funnel with a small amount of water and add 10 mL of saturated sodium chloride solution. Extract three times with 50 mL of n-hexane (a free form of fluthiacet-methyl is present in the aqueous layer; see Section 8). Dry the n-hexane extract through 80 g of anhydrous sodium sulfate on a glass funnel and transfer the dried extract into a 300-mL separatory funnel. Extract twice with 70 mL of acetonitrile, collect the extract in a 300-mL round-bottom flask, and evaporate the solvent under reduced pressure. Dissolve the residue in 10 mL of n-hexane–ethyl acetate (4 : 1, v/v), transfer the solution to a Bond Elut LRC SI column and discard the first eluate. Connect a Sep-Pak Plus NH_2 cartridge to the outlet of a Bond Elut LRC SI cartridge and elute fluthiacet-methyl with 15 mL of n-hexane–ethyl acetate (2 : 3, v/v). Collect the eluate in a 50-mL pear-shaped flask, evaporate the solvent under reduced pressure and dissolve the residue in an appropriate volume of acetone for analysis.

6.2.2 Soil

Transfer the concentrate from Section 6.1.2 into a 200-mL separatory funnel with a small amount of water and extract three times with 30 mL of n-hexane (a free form of fluthiacet-methyl is present in the aqueous layer; see Section 8). Dry the n-hexane extract with anhydrous sodium sulfate. Transfer the n-hexane extract to a Sep-Pak Plus silica cartridge and elute the solution. Wash the container with 10 mL of n-hexane, transfer the washings to the cartridge and elute the solution. Then wash the cartridge sequentially with 20 mL of n-hexane and with 6 mL of n-hexane–ethyl acetate (4 : 1, v/v). Discard all of these eluates. Elute fluthiacet-methyl in the cartridge with 15 mL of n-hexane–ethyl acetate (2 : 3, v/v). Collect the eluate in a 50-mL pear-shaped flask and evaporate the solvent under reduced pressure. Dissolve the residue in 5 mL of acetonitrile–toluene (3 : 1, v/v) and transfer the solution to the Supelclean Envi-Carb + LC-NH_2 column. Wash the container twice with 5 mL of the mixture and transfer the washings to a cartridge, then wash the cartridge with 5 mL of the same solvent. Collect the eluate in a 50-mL pear-shaped flask and evaporate the solvent under reduced pressure. Dissolve the residue in an appropriate volume of acetonitrile–water (7 : 3, v/v) for analysis.

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6.2.3 Water

Dry the dichloromethane extract from Section 6.1.3 with anhydrous sodium sulfate and collect the solution in a 1-L round-bottom flask. Evaporate the solvent at 37 °C under reduced pressure. Dissolve the residue in 4 mL of acetone–n-hexane (1 : 9, v/v) and adsorb on the top of a silica gel column bed. Rinse the flask three times with 1 mL of the solvent mixture and transfer the rinsings to the column. Elute interfering substances with 85 mL of the solvent mixture and discard the eluate. Then elute fluthiacet-methyl with 140 mL of the solvent mixture. Collect the eluate in a 300-mL

pear-shaped flask and evaporate to dryness under reduced pressure at 37 °C. Dissolve the residue in an appropriate volume of the solvent mixture for analysis.

6.3 Determination

6.3.1 Gas-chromatographic determination

Inject an aliquot (V_i) of the solution derived from Section 6.2.1 (V_{End}) for crops and Section 6.2.3 for water (V_{End}) into the gas chromatograph.

Operating conditions for crops

Gas chromatograph	Shimadzu GC-17A
Column	HP-1 MS, 0.32-mm i.d., 30-m length, 0.25-μm film thickness (Hewlett-Packard)
Column temperature	280 °C
Injection port	Splitless capillary inlet, temperature 290 °C
Injection port pressure	AFC system, initial 26 psi, 0.5 min 46 psi (+40 psi min^{-1}), 1.5 min 26 psi (−40 psi min^{-1})
Detector	Flame thermionic detector, temperature 310 °C
Gas flow rates	Helium, carrier gas, 3.1 mL min^{-1} Makeup gas 30 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 100 mL min^{-1}
Injection volume	2 μL
Retention time	4.4 min
Minimum detectable amount	0.6 ng

Operating conditions for water

Gas chromatograph	Hitachi 163
Column	DB-1, 0.53-mm i.d., 20-m length, 1.5-μm film thickness (J&W Scientific)
Column temperature	260 °C
Injection port temperature	280 °C
Detector	Electron capture detector (^{63}Ni, 10 mCi, pulse interval 100 μs), temperature 280 °C
Gas flow rate	Nitrogen carrier gas, 28 mL min^{-1}
Attenuation	32 (range 10)
Injection volume	2 μL
Retention time	5.0 min
Minimum detectable amount	0.02 ng

6.3.2 High-performance liquid chromatographic determination

Inject an aliquot (V_i) of the solution derived from Section 6.2.2 (V_{End}) for soils into the high-performance liquid chromatograph.

Operating conditions for soil

Instrumentation	Shimadzu LC-10A Shimadzu LC-10AD detector, Shimadzu C-R6A integrator
Column	TSK-gel ODS 120T, 4.6-mm i.d., 25-cm length (TOSOH)
Mobile phase	Acetonitrile–water (7 : 3,v/v)
Flow rate	1.0 mL min^{-1}
Wavelength	254 nm
Attenuation	0.01 ABS
Injection volume	20 μL
Retention time	7.7 min
Minimum detectable amount	1.0 ng

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Construct a fresh calibration curve with fluthiacet-methyl standard solutions for each set of analyses. Using log–log paper, plot the peak heights in millimeters against the injected amount of fluthiacet-methyl in nanograms. Measure the peak heights of fluthiacet-methyl on a chromatogram of a sample extract, and quantify fluthiacet-methyl by comparing the peak height with the calibration curve.

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7.2 Recoveries and limit of detection

The recoveries from untreated control samples fortified with fluthiacet-methyl at 0.2 mg kg^{-1} were 96% [relative standard deviation (RSD) 3.1%] for corn and 74% (RSD 8.0%) for green corn. The limit of detection was 0.01 mg kg^{-1}.

The recoveries from untreated control soils fortified with fluthiacet-methyl at 0.1 and 0.2 mg kg^{-1} were 85–103% and 87–103%, respectively. The limit of detection was 0.01 mg kg^{-1}.

The recovery from water samples fortified with fluthiacet-methyl at 2.0 μg kg^{-1} was 92%. The limit of detection was 0.05 μg kg^{-1}.

7.3 Calculation of residues

The residue R, expressed in milligrams per kilogram of fluthiacet-methyl, is calculated with the following equation:

$$R = (W_A \times V_{End})/(V_i \times G)$$

where

G = sample weight (g)
V_{End} = terminal volume of sample solution from Section 6.2 (mL)
V_i = portion of volume of V_{End} injected into the GC or HPLC system (μL)
W_A = amount of fluthiacet-methyl for V_i read from calibration curve (ng)

8 Important points

Fluthiacet-methyl is rapidly degraded in soil (DT_{50}, 1.0–1.3 h in soil) to produce [2-chloro-4-fluoro-5-(5,6,7,8-tetrahydro-3-oxo-1*H*,3*H*-[1,3,4] thiadiazolo[3,4-*a*]pyridazin-1-ylidenamino)phenylthio]acetic acid (abbreviated as the free form), as the main degradation product, due to hydrolysis of methyl ester. Since it is degraded by soil microorganisms and by other degradation environments, soil samples should be analyzed immediately after sampling. In addition, soil samples should be frozen immediately after sampling. In the sample extract prepared by this analysis method, a main degradation product, free form, is also extracted simultaneously, and there are no interfering substances on simultaneous analysis. The procedures for the simultaneous analysis of fluthiacet-methyl and its free form are outlined as follows.

After recovering fluthiacet-methyl from the crop extract with n-hexane, acidify the residual aqueous layer and extract the free form of fluthiacet-methyl with n-hexane–ethyl acetate (2 : 1, v/v). After evaporating the solvent, clean up the residue with an C_{18} Empore Disk Cartridge. After methylation of the free form with trimethylsilyldiazomethane, clean up the ester with a Bond Elut LRC SI and a Sep-Pak Plus NH_2 cartridge, and quantify as fluthiacet-methyl by GC/FTD.

After extracting fluthiacet-methyl from the soil extract with n-hexane, pass the residual aqueous layer through a dual cartridge of Sep-Pak Plus NH_2 and Sep-Pak Plus C_{18} to adsorb the free form of fluthiacet-methyl on Sep-Pak Plus C_{18}. Remove the Sep-Pak Plus C_{18}, wash it with 0.5% acetic acid and acetonitrile–water–acetic acid (20 : 80 : 0.5, v/v/v), elute with acetonitrile–water–acetic acid (50 : 50 : 0.5, v/v/v) and quantify the free form by HPLC. The operating conditions for HPLC are the same as those for fluthiacet-methyl, except that the mobile phase is acetonitrile–water–acetic acid (50 : 50 : 0.5, v/v/v) (retention time 8.8 min).

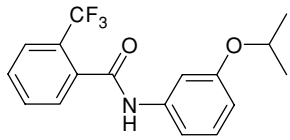
The recovery of free form is 86% (RSD 8%) for 0.2 mg kg^{-1}-fortified corn and 80% (RSD 4%) for 0.2 mg kg^{-1}-fortified soil.

Mitsumasa Ikeda, Yoshihiro Saito and Akira Yagi
Kumiai Chemical Industry Co. Ltd, Shizuoka, Japan

Flutolanil

Materials to be analyzed	Plants (rice, potato, cabbage, lettuce, soybean, pear, cucumber, tomato, eggplant, ornamentals), soil and water
Instrumentation	Gas-chromatographic determination [with mass spectrometry (MS) or flame thermionic detection (FTD)] for plant materials, soil and water

1 Introduction

Chemical name (IUPAC)	α,α,α-Trifluoro-3′-isopropoxy-*o*-toluanilide
Structural formula	
Empirical formula	$C_{17}H_{16}F_3NO_2$
Molar mass	323.3
Melting point	100.4–103.8 °C
Vapor pressure	6.5×10^{-6} Pa at 25 °C
Solubility	Water 6.53 mg L^{-1} at 20 °C Acetone 1439 g L^{-1} at 25 °C Methanol 832 g L^{-1} at 25 °C Ethanol 374 g L^{-1} at 25 °C Chloroform 674 g L^{-1} at 25 °C Benzene 135 g L^{-1} at 25 °C *p*-Xylene 29 g L^{-1} at 25 °C Ethyl acetate 675 g L^{-1} at 25 °C
Stability	Stable in acidic and alkaline media (pH 3–11) Stable to heat and light
Other properties	Log $K_{ow} = 3.7$
Use pattern	Flutolanil is a benzanilide fungicide discovered and developed by Nihon Nohyaku Co., Ltd.[1] Flutolanil exhibits excellent control against the following pathogens at rates of 300–1000 g ha^{-1} (foliar spray), 1.5–3.0 g kg^{-1} (seed treatment) and 2.5–10.0 kg ha^{-1} (soil incorporation): *Rhizoctonia solani* on rice,

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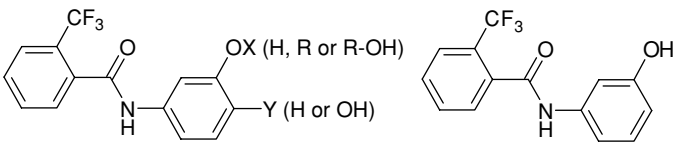
Typhula spp. and *R. cerealis* on cereals, *R. solani* on potatoes, *Corticium rolfsii* and *R. solani* on sugar beet, *R. solani* on vegetables, *Sclerotium rolfsii* on peanuts, *Gymno-sporangium* spp. on pome fruit, *R. solani* and *S. rolfsii* on ornamentals, and *R. solani*, *S. rolfsii*, *R. cerealis* and *Lepista* spp. on turf.

Flutolanil is an inhibitor of succinate dehydrogenase complex (Complex II), in the mitochondrial respiratory electron transport chain.[2]

This systemic fungicide has both protective and curative properties for preventing fungal growth and penetration from infection cushions, causes collapse of hyphae and infection cushions.

Regulatory position

The major metabolic pathways of flutolanil in plants are *para*-hydroxylation of the aniline ring and hydroxylation of the isopropoxy side chain. For potatoes, flutolanil and desisopropyl-flutolanil (M-4) are selected as the target analytes. For rice plant, other metabolites containing 2-(trifluoromethyl)benzanilide moiety are also selected as the target analytes. For soil and water samples, flutolanil is selected as the only target analyte.



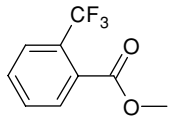
Typical metabolites of flutolanil

2 Outline of method

Four different types of residue analytical methods of flutolanil and its metabolites are developed for plant (potato and rice), soil and water:

A *'Multi-residue analytical method'* is provided for potatoes.
Flutolanil and its metabolite M-4 (desisopropyl-flutolanil) are methylated and analyzed simultaneously using gas chromatography/mass spectrometry (GC/MS).

B *'Total toxic residue analytical method'* is provided for rice plants.
Flutolanil and its metabolites containing the 2-(trifluoromethyl)benzanilide moiety are converted to methyl 2-(trifluoromethyl)benzoate (2-TFBA Me-ester) by alkaline hydrolysis and methylation for GC/MS analyses.



2-TFBA Me-ester

C *'GC/FTD Method'* determines flutolanil in soil samples.
D *'GC/MS Method'* determines flutolanil in water samples.

3 Multi-residue analytical method (for potatoes)

3.1 Apparatus

Homogenizer (Ultra Turrax or equivalent)
Centrifuge (Hettich Rotixa or equivalent)
Chromatographic columns (glass with stopcock and solvent reservoir, 10-mm i.d.)
Fused-silica capillary column, DB-1701, 60 m × 0.32-mm i.d., 0.15-μm film thickness (14% cyanopropylphenyl)methylpolysiloxane
Varian 3400 gas chromatograph equipped with a temperature-programmed SPI injector, a Varian 8100 autosampler, and a Varian Saturn II Iontrap mass spectrometer
Centrifuge vials, 10- and 250-mL
Evaporation flasks, 100- and 250-mL
Separatory funnel, 250-mL

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3.2 Reagents and supplies

Acetone, reagent grade
n-Hexane, reagent grade
Toluene, reagent grade
Dichloromethane, reagent grade
Ethyl acetate, reagent grade
Sodium chloride, reagent grade
Celite (Fluka)
Dimethyl sulfoxide (DMSO), reagent grade
Methyl iodide, reagent grade
Silica gel (Merck)
Sodium hydroxide, reagent grade

3.3 Procedure

3.3.1 Extraction

Weigh 50 g of the potato sample into a 250-mL centrifuge vial and homogenize with 100 mL of acetone. After centrifugation at 1500–2000 rpm for 5 min, decant the supernatant over filter paper filled with Celite and collect in a 500-mL evaporation flask. Repeat extraction with 80 mL of acetone, centrifuge, filter and combine the extracts. Rinse Celite with 20 mL of acetone into the combined filtrates.

3.3.2 Cleanup

Partitioning

Concentrate the extracts to about 40 mL at 60 °C under reduced pressure in a rotary evaporator. Transfer the concentrate into a 250-mL separatory funnel, rinse the evaporation flask with 100 mL of aqueous 10% sodium chloride solution and then with 50 mL of dichloromethane, and add the rinsates to the separatory funnel. After shaking the separatory funnel, filter the lower organic phase over anhydrous sodium sulfate into a 250-mL evaporation flask. Repeat partitioning with a second portion of 50 mL of dichloromethane and combine the organic phases. Evaporate the solvent with a rotary evaporator at 40 °C.

Derivatization/partitioning

Transfer the residue with 2 × 1 mL of DMSO into a 10-mL centrifuge vial. Add 20 µL of methyl iodide and 200 µL of 1 M sodium hydroxide solution, mix and plug the vial with a stopper. Derivatization is done at 70 °C for 1 h. After addition of 45 mL of aqueous 10% sodium chloride, extract the reaction mixture with 10 or 2 × 10 mL of n-hexane. Transfer the n-hexane phase on to the chromatographic column prepared as described below.

Column chromatography

Prepare a chromatographic column with silica gel (5 g, deactivated with 1.5% water) packed in n-hexane. Add a 3-cm layer of anhydrous sodium sulfate on the top of the silica gel column. Drain the n-hexane down to the sodium sulfate layer. Transfer the n-hexane phases obtained from the derivatized samples into the column and let the n-hexane drain. Elute with a first fraction of n-hexane–ethyl acetate (50 mL, 9 : 1, v/v) and discard the eluate. Elute with a second fraction of n-hexane–ethyl acetate (50 mL, 7 : 3, v/v) and collect the eluate in a 100-mL evaporation flask. Concentrate the eluate to dryness with a rotary evaporator and dissolve the residue in 5 mL of toluene for GC/MS analyses.

3.3.3 Determination

Inject an aliquot of the gas chromatography (GC)-ready sample solution into the GC/MS system.

Operating conditions for GC/MS

Gas chromatograph	Model 3400, Varian
Sample injector	Splitless mode
Column	Fused-silica capillary column, DB-1701, 60 m × 0.32-mm i.d., 0.15-µm film thickness
Column temperature	Initial 90 °C, held for 2 min, increased at 30 °C min^{-1} to 230 °C, then at 3 °C min^{-1} to 250 °C and at 30 °C min^{-1} to 300 °C, held for 7 min
Injection port temperature	Initial 120 °C, held for 0.1 min, increased at 200 °C min^{-1} to 260 °C, held for 1 min

Detector	Electron impact MS detector, Saturn II Iontrap MS
Selected ion monitoring	Narrow mass range *m/z* 140–190 *m/z* 145, 173
Gas flow rate	Helium carrier gas, head pressure set to 30 psi
Injection volume	1 μL

3.4 Evaluation

3.4.1 Method

Standardization

Peaks of the *N*-methyl derivative of flutolanil and *N*,*O*-dimethyl derivative of the metabolite M-4 usually appear at retention times around 11.4 and 11.0 min, respectively. Plot the peak areas against the amounts of the analytes.

Detection of sample residues

Inject the cleaned up sample into the GC/MS system operated under the same conditions as employed for standardization. Compare the peak areas of the analytical samples with the calibration curve. Determine the concentrations of the *N*-methyl derivative of flutolanil and *N*,*O*-dimethyl derivative of the metabolite M-4 present in the sample.

3.4.2 Recoveries, limit of detection and limit of determination

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With fortification levels of 0.01–0.1 mg kg^{-1}, average recoveries of flutolanil and the metabolite M-4 from untreated potato are 80 and 76%, respectively.
The limit of quantitation is 0.01 mg kg^{-1}.

Calculate the concentrations of the analytes (flutolanil and M-4) in potato samples (mg kg^{-1}) with the following equation:

$$\text{Analyte concentration} = \frac{A \times V}{W}$$

where

A = concentration of the analyte in the final solution (μg mL^{-1})
V = volume of the final solution (mL)
W = weight of analysis sample (g)
F = molecular weight factor: flutolanil/*N*-methyl-flutolanil = 0.958
M-4/*N*,*O*-dimethyl-M-4 = 0.909

4 Total toxic residue analytical method (for rice plant)

4.1 Apparatus

Whatman cellulose extraction thimbles (33 × 94-mm)
Soxhlet extraction tubes (400-mm)

Glass wool (silane-treated)
Reflux condensers (300-mm)
Rotary evaporator
Heating mantles
Round-bottom flasks, 125- and 500-mL
Teflon culture tubes, 15-mL
Teflon tape
Heating block, Reacti-Therm III with Reacti-Vap Evaporator or equivalent
Culture tubes with screw-caps, 50-mL
Volumetric flasks, 10-mL
Diolsilane solid-phase extraction (SPE) cartridges, 3-mL (J.T. Baker Inc.)
Aminopropyl SPE cartridges, 3-mL (J.T. Baker Inc.)
Filtration columns, 6-mL (J.T. Baker Inc.)
Solvent reservoirs, 75-mL
Fused-silica capillary column, DB-17, 30 m × 0.25-mm i.d., 0.25-μm film thickness, (50% phenyl)–methylpolysiloxane
Hewlett-Packard 5890A gas chromatograph with capillary split/splitless inlet with HP5970B mass-selective detector equipped with a Model 7673A autosampler
Hobart VCM-40 or other apparatus suitable for grinding frozen plant tissue

4.2 Reagents and supplies

Acetone, pesticide grade or equivalent
Acetonitrile, pesticide grade or equivalent
n-Hexane, pesticide grade or equivalent
Toluene, pesticide grade or equivalent
Boiling chips
Olive oil, reagent grade
Sodium hydroxide (50%, w/w)
Sulfuric acid, concentrated, reagent grade
Sodium sulfate, granular anhydrous, reagent grade
Citric acid, reagent grade
Nitrogen, UPC grade or equivalent
Diazomethane, reagent grade
Diethylene glycol, reagent grade
Potassium hydroxide, reagent grade
Methanol, reagent grade

4.3 Procedure

4.3.1 Extraction

Weigh a finely ground representative crop sample (20 g for grain or 10 g for straw) into a cellulose extraction thimble. Assemble a Soxhlet extractor using a 500-mL round-bottom flask containing 200 mL of acetone and boiling chips. Place the extraction

thimble containing the sample in the Soxhlet extractor. Place a plug of glass wool in the cellulose extraction thimble and attach a reflux condenser to the extractor. Extract the crop material overnight with the Soxhlet extractor. Turn off the Soxhlet extraction unit and allow it to cool to room temperature. Rinse the condensers with acetone and collect the rinsings in a 500-mL round-bottom flask.

4.3.2 Cleanup

Solvent partitioning

Concentrate the extracts to dryness at 40 °C under reduced pressure with the rotary evaporator and reconstitute with 2 × 15 mL of acetonitrile. Partition the solution with 2 × 15 mL of n-hexane and collect the acetonitrile phase in a 125-mL round-bottom flask.

Base hydrolysis

Concentrate the acetonitrile extracts obtained above to dryness below 40 °C with the rotary evaporator. Dissolve the residues in 2 mL of acetone. Quantitatively transfer the acetone extracts to a culture tube with a Teflon screw-cap containing 250 μL of acetone–olive oil keeper (1 : 1, v/v). Evaporate the acetone on a heating block not exceeding 40 °C under a stream of air. Wrap the threads on the Teflon culture tube with Teflon tape and add 2.0 mL of 50% (w/w) sodium hydroxide. Cap tightly and heat the Teflon culture tube at approximately 200 °C for 3 h.

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Diol SPE cleanup

Quantitatively transfer the hydrolysis reaction solution to a 50-mL glass culture tube with a screw-cap by rinsing with 3 × 5 mL of deionized water followed by 5 mL of 30% (v/v) sulfuric acid and one additional 5 mL of deionized water. Rinse the Teflon culture tube with acetone and transfer to the glass culture tube. Extract the acidic aqueous phase (pH 1) with 3 × 2.5 mL of toluene. Pass each upper toluene phase through approximately 3 g of anhydrous sodium sulfate contained in a 6-mL disposable filtration cartridge into a 10-mL volumetric flask. Adjust the volume of the solution to 10 mL with toluene. Condition a 3-mL diolsilane bonded silica gel SPE cartridge with two column volumes of toluene. Load a 5-mL aliquot of toluene solution and collect the eluate in a 125-mL round-bottom flask. Elute the column with an additional 50 mL of toluene (use the 75-mL reservoirs) and collect the eluate in the same round-bottom flask. Concentrate the toluene extract to approximately 3.0 mL at 40 °C under weak reduced pressure with a rotary evaporator.

Methylation

Prepare sufficient diazomethane to methylate the sampled as required. Add the diazomethane prepared in toluene (3 mL) to the extract. Seal the flask with a stopper and gently swirl the reaction mixture. Leave the reaction mixture at room temperature for

30 min. Quantitatively transfer the reaction mixture above into a 10-mL volumetric flask and dilute to 10 mL with toluene. Remove any excess diazomethane from the reaction mixture by the addition of 500 μL of 0.1 N citric acid solution to the reaction mixture above. Seal the volumetric flask with a stopper, shake well and allow the aqueous phase to settle out.

Amino SPE cleanup

Place approximately 0.75 g of anhydrous sodium sulfate in an unconditioned 3-mL aminopropyl-bonded SPE cartridge. Transfer an aliquot (2 mL) from the above toluene solution to the aminopropyl SPE cartridge and collect the eluate in a GC vial for analysis by GC/MS.

4.3.3 Determination

Inject an aliquot of the GC-ready sample solution into the GC/MS system.

Operating conditions for GC/MS

Gas chromatograph	Model GC5890A, Hewlett-Packard
Sample injector	Split mode
Column	Fused-silica capillary column, DB-17, 30 m × 0.25-mm i.d., 0.25-μm film thickness
Column temperature	Initial 150 °C, held for 2 min, increased at 10 °C min^{-1} to 250 °C, no hold
Injection port temperature	150 °C
Detector	Mass-selective detector, MSD5970B Temperature 250 °C
Selected ion monitoring	*m/z* 145, 173
Gas flow rate	Helium carrier gas, head pressure set to 10 psi
Injection volume	2 μL

4.4 Evaluation

4.4.1 Method

Standardization

The peak of 2-TFBA Me-ester usually appears at a retention time around 2.4 min. Construct a calibration curve by plotting the natural logarithm of the peak area counts against the natural logarithm of the standard concentration to obtain a least-squares regression line.

Detection of sample residues

Inject a 2-μL aliquot of the cleaned-up sample into the GC/MS system operated under the same conditions as employed for standardization.

Compare the peak areas of the analytical samples with the calibration curve. Determine the concentration of 2-TFBA Me-ester present in the sample using the following

equation:

$$\text{2-TFBA Me-ester concentration} = \frac{\exp[(\ln A - Y/S)]}{C}$$

where

A = peak area (m/z 145 + m/z 173) for 2-TFBA Me-ester
Y = y-intercept of calibration curve [ln(area counts)]
S = slope of calibration curve {ln[area counts/(μg mL^{-1})]}
C = crop solvent ratio (g mL^{-1})

4.4.2 Recoveries, limit of detection and limit of determination

With a fortification level of 0.10 mg kg^{-1}, the average recovery of flutolanil and the metabolites from untreated rice grain is 76%.
With a fortification level of 5.00 mg kg^{-1}, the average recovery of flutolanil and the metabolites from untreated rice straw is 80%.
The limit of determination is 0.05 mg kg^{-1}.

4.4.3 Calculation of residues

Calculate the total residue in terms of total flutolanil in plant materials (mg kg^{-1}) with the following equation:

$$\text{Total flutolanil concentration} = B \times F$$

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where

B = concentration of 2-TFBA Me-ester (mg kg^{-1})
F = flutolanil molecular weight factor (1.584)

4.5 Important points

In the 'Total toxic residue analytical method', careful handling is recommended for concentrating steps to prevent the loss of volatile 2-TFBA and 2-TFBA Me-ester.

5 GC/FTD method

5.1 Apparatus

Shimadzu GC-7A gas chromatograph with flame thermionic detector
Pyrex spiral column packed with 3% OV-1 on Gas Chrom Q (80–100 mesh), 1 m × 3-mm i.d.
Rotary evaporator
Shaker (Iwaki Co., Model KM or equivalent)
Erlenmeyer flask, 500-mL
Separatory funnel, 200-mL

5.2 Reagents and supplies

Acetone, reagent grade
Ethyl acetate, reagent grade
n-Hexane, reagent grade
Sodium sulfate, granular anhydrous, reagent grade
Sodium chloride, reagent grade
Whatman No. 2 filter paper
Florisil PR (Wako Pure Chemical Co.) or equivalent
Hyflo Super-Cel (Johns-Manville Co.) or equivalent

5.3 Procedure

5.3.1 Extraction

Weigh 50 g (dry weight) of soil sample into a 500-mL Erlenmeyer flask. Add 250 mL of acetone–water (4 : 1, v/v) and shake vigorously for 1 h and filter the solution by suction through Whatman No. 2 filter paper covered with a 1-cm layer of Hyflo Super-Cel. Rinse the flask and cake with 100 mL of acetone. Combine the filtrate and rinsings and concentrate to less than 40 mL below 40 °C with a rotary evaporator.

5.3.2 Cleanup

Transfer the concentrate into a 200-mL separatory funnel using two portions of 20 mL of n-hexane. Add 100 mL of saturated sodium chloride aqueous solution and extract twice with 100 mL of n-hexane by shaking for 5 min and allow the phases to separate. After dehydration of the n-hexane extract with 10 g of anhydrous sodium sulfate, concentrate the extract to dryness below 40 °C with a rotary evaporator. Transfer the residue with three portions of 5 mL of n-hexane into a glass column containing 10 g of Florisil (deactivated by water at a rate of 1%). Elute with 100 mL of n-hexane–ethyl acetate (9 : 1, v/v) and then with 100 mL of n-hexane–ethyl acetate (7 : 3, v/v). Concentrate the second eluate to dryness and dissolve the residue in 10 mL of n-hexane and analysis by gas chromatography/flame thermionic detection (GC/FTD).

5.3.3 Determination

Inject an aliquot of the GC-ready sample solution into the GC/FTD system.

Operating conditions for GC/FTD

Gas chromatograph	Model GC7A, Shimadzu
Column	Pyrex spiral column packed with 3% OV-1 on Gas Chrom Q (80–100 mesh), 1 m × 3-mm i.d.
Column temperature	220 °C, constant
Injection port temperature	250 °C
Detector temperature	250 °C
Detector	Flame thermionic detector

Gas flow rates	Helium carrier gas, 40 mL min^{-1} Hydrogen, 2 mL min^{-1} Air, 120 mL min^{-1}
Injection volume	2 μL

5.4 Evaluation

5.4.1 Method

Standardization

The peak of flutolanil usually appears at a retention time around 3.2 min. Plot the peak areas against the amounts of flutolanil.

Detection of sample residues

Inject a 2-μL aliquot of the cleaned-up sample into the GC/FTD system operated under the same conditions as employed for standardization. Compare the peak areas of the analytical samples with the calibration curve. Determine the concentration of flutolanil present in the sample.

5.4.2 Recoveries, limit of detection and limit of determination

With fortification levels of 0.1 and 1 mg kg^{-1}, the recoveries of flutolanil from untreated soils are 98 and 95%.
The limit of detection is 0.01 mg kg^{-1}.

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5.4.3 Calculation of residues

Calculate the concentrations of flutolanil in soils (mg kg^{-1}) with the following equation:

$$\text{Flutolanil concentration} = \frac{A \times V}{W}$$

where

A = concentration of the analyte in the final solution (μg mL^{-1})
V = volume of the final solution (mL)
W = weight of analysis sample (g)

6 GC/MS method

6.1 Apparatus

Rotary evaporator
Hewlett-Packard 5890 Series II gas chromatograph with capillary split/splitless inlet with HP5971A mass-selective detector equipped with a Model 7673 autosampler

Fused-silica capillary column, HP-5, 30 m × 0.25-mm i.d., 0.25-μm film thickness, (5% phenyl)–methylpolysiloxane

6.2 Reagents and supplies

Ethyl acetate, reagent grade
Acetone, reagent grade
Methanol, reagent grade
Distilled water, reagent grade
C_{18} SPE cartridge column, Sep-Pak Plus PS-2 (Waters)

6.3 Procedure

6.3.1 Extraction

Measure a 1000 mL aliquot of sample solution and transfer to the top of the C_{18} SPE cartridge column which was conditioned with 5 mL of ethyl acetate, 5 mL of methanol and 10 mL of distilled water in advance at an elution rate of 10 mL min^{-1}. After washing the column with 10 mL of distilled water, dry the column with suction for 30 min, then elute with 0.5 mL of acetone and then 5 mL of ethyl acetate. Dry the eluates using a rotary evaporator at 35 °C and by N_2 purging.

6.3.2 Cleanup

No further cleanup procedure is needed for the water sample.

6.3.3 Determination

Dissolve the residue in 0.5 mL of acetone and inject a 2-μL aliquot of solution into the GC/MS system.

Operating conditions for GC/MS

Gas chromatograph	Model GC5890 Series II, Hewlett-Packard
Sample injector	Splitless mode
Column	Fused-silica capillary column, HP-5, 30 m × 0.25-mm i.d., 0.25-μm film thickness
Column temperature	Initial 150 °C, held for 4 min, increased at 20 °C min^{-1} to 220 °C, held for 4.5 min, increased at 10 °C min^{-1} to 230 °C, and then at 30 °C min^{-1} to 250 °C, held for 1.5 min
Injection port temperature	250 °C
Detector	Mass-selective detector, MSD5971A Temperature 280 °C
Selected ion monitoring	Target ion: *m*/*z* 173 Reference ion: *m*/*z* 323
Gas flow rate	Helium carrier gas, 0.6 mL min^{-1}
Injection volume	2 μL

6.4 Evaluation

6.4.1 Method

Standardization

The peak of flutolanil usually appears at a retention time around 12.5 min. Plot the peak areas against the amounts of flutolanil.

Detection of sample residues

Inject a 2-μL aliquot of the cleaned-up sample into the GC/MS system operated under the same conditions as employed for standardization. Compare the peak areas of the analytical samples with the calibration curve. Determine the concentration of flutolanil present in the sample.

6.4.2 Recoveries, limit of detection and limit of determination

With fortification levels between 0.05 and 0.5 mg L^{-1}, the recoveries from blank distilled water ranged from 86 to 98% with the limit of quantitation being 0.05 mg L^{-1}.

6.4.3 Calculation of residues

Calculate the concentrations of flutolanil in water (mg L^{-1}) with the following equation:

$$\text{Flutolanil concentration} = \frac{A \times V}{W}$$

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where

A = concentration of the analyte in the final solution (μg mL^{-1})
V = volume of the final solution (mL)
W = volume of analysis sample (mL)

References

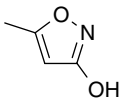
1. F. Araki and K. Yabutani, 'α,α,α-Trifluoro-3′-isopropoxy-*o*-toluanilide (NNF-136): a new fungicide for the control of diseases caused by Basidiomycetes,' in "Proceedings of 1981 British Crop Protection Conference on Pests and Diseases," Vol. 1, pp. 3–9 (1981).
2. K. Motoba, M. Uchida, and E. Tada, *Agric. Biol. Chem.,* **52**, 1445 (1988).

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Hymexazol

Materials to be analyzed	Brown rice, watermelon, cucumber, sugar beat, spinach, pea and tobacco
Instrumentation	Gas-chromatographic determination for plant materials

1 Introduction

Chemical name (IUPAC)	5-Methylisoxazol-3-ol
Structural formula	
Empirical formula	$C_4H_5NO_2$
Molar mass	99.1
Melting point	86–87 °C
Boiling point	202 ± 2 °C
Vapor pressure	182 mPa at 25 °C
Solubility	Water: 65.1 (pure), 58.2 (pH 3), 67.8 (pH 9) g L^{-1} at 20 °C Readily soluble in organic solvents
Stability	Stable under alkaline conditions and relatively stable under acidic conditions.
Use pattern	Hymexazol is used for the control of soil-borne diseases for rice, sugar beet, spinach, pea, cucumber, watermelon, grass, etc. Applied as a soil drench or by soil incorporation and used as a seed dressing for sugar beet. Hymexazol also exhibits some plant growth stimulation activity.
Regulatory position	The definition of residue is for the parent, hymexazol, only.

2 Outline of method

Hymexazol residues are extracted from plant materials with acetone and partitioned into aqueous sodium hydrogen carbonate solution. The aqueous solution is washed with dichloromethane and diethyl ether. After acidification of the

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aqueous phase, hymexazol is extracted with diethyl ether. The amount of hymexazol is directly determined by gas chromatography (GC) with nitrogen–phosphorus detection.

3 Apparatus

Homogenizer (Cooking cutter, Hitachi, Japan)
Erlenmeyer flask, 300-mL
Round-bottom flask, 300-mL
Shaker: Iwaki K-M mechanical shaker type V-D (Iwaki Seisakusho Co., Japan)
Gas chromatograph: Hewlett-Packard Model 6890 equipped with a nitrogen–phosphorus flame ionization detector
Capillary column for gas–liquid chromatography (GLC), DB-WAX, 0.53-mm i.d. × 15-m long, 1-μm film thickness (J&W Scientific, USA)
Glass funnel with glass filter, 17G-3
Solvent evaporation system (rotary evaporator, water-bath and vacuum line)

4 Reagents

Hymexazol: analytical standard, recrystallized at least four times from hexane
Acetone, diethyl ether, dichloromethane: reagent grade for residue analysis
Hydrochloric acid, sodium hydrogen carbonate, sodium chloride, potassium carbonate sodium sulfate anhydrous: reagent grade
Polyethylene glycol 400, average molecular weight 380–420: gas chromatography grade

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5 Sampling and preparation

Samples are chopped and homogenized with a homogenizer for 5 min and should then be extracted quickly with organic solvents as described in the following procedure.

6 Procedure

6.1 Extraction

Weigh 20 g (fresh weight) of chopped and homogenized plant samples into a 300-mL Erlenmeyer flask. Add 80 mL of acetone and shake the flask vigorously for 30 min with a shaker. In the case of brown rice and pea, add 20 mL of water to 10 g of sample and allow to stand for 2 h before adding 80 mL of acetone. Filter the extraction mixture by suction through a glass filter and re-extract the residue on the filter with 50 mL of acetone, then filter the mixture by suction. Concentrate the combined filtrate in the 300-mL of round-bottom flask to remove acetone at below 30 °C after addition of a 25% aqueous solution of potassium carbonate (0.2 mL).

6.2 Liquid–liquid partition

Transfer the whole volume of the aqueous solution in to a 200-mL separatory funnel with the aid of distilled water (30 mL, in small portions). Add 1 g of sodium hydrogen carbonate and ca 7 g of sodium chloride to the funnel to saturate the solution. Wash the solution twice with 50 mL of dichloromethane and twice with 50 mL of diethyl ether and discard the washings. Add 2 N hydrochloric acid to the aqueous solution to adjust the acidity to pH 2–3. Extract the aqueous solution three times with 50 mL of diethyl ether. Combine and dry the diethyl ether extracts by passing through 50 g of anhydrous sodium sulfate into a 300-mL round-bottom flask. After adding 1 mL of a 2% acetone solution of polyethylene glycol, evaporate the majority of the diethyl ether below 30 °C. Finally, evaporate the remaining diethyl ether under a stream of nitrogen at room temperature to prepare the cleaned-up sample for GC analysis.

6.3 Determination by gas chromatography

Dissolved the cleaned-up sample in 4 mL of acetone and inject a 2-μL portion of the sample solution into the preconditioned gas chromatograph. Determine the concentration of hymexazol by relating the height of the hymexazol peak to a fresh calibration curve.

Operating conditions

Gas chromatograph	Hewlett-Packard Model 6890 equipped with a nitrogen–phosphorus flame ionization detector
Packed column	Capillary column for GLC, DB-WAX, 0.53-mm-i.d. × 15-m long, 1-μm film thickness
Column temperature	140 °C, held for 5 min, increased at 15 °C min^{-1} to 200 °C, held for 6 min
Injection port temperature	140 °C
Detector temperature	250 °C
Gas flow rates	Helium carrier gas, 4.2 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 60 mL min^{-1}
Injection volume	2 μL
Retention time	10 min

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. A fresh calibration curve is constructed with hymexazol standard solutions. The calibration curve is plotted as peak height versus the amount of hymexazol injected.

7.2 Limit of detection

The limit of detection of hymexazol in vegetables is 0.01 mg kg^{-1}, as shown below.

Minimum detectable amount: 0.1 ng
Detection limit = (0.1 ng × 4 mL)/(2 µL × 20 g) = 0.01 mg kg^{-1}
Sample volume injected (V_i): 2 µL
Final solution volume (V_{End}): 4 mL
Sample weight (G): 20 g
Detected amount (W)
The residue R, expressed as mg kg^{-1} hymexazol, is calculated from the following equation:

$$R = \frac{W \times V_{End}}{V_i \times G}$$

where

G = sample weight (g)
V_{End} = terminal volume of sample solution from Section 6.2 (mL)
V_i = portion of volume of V_{End} injected into the gas chromatograph (µL)
W = amount of hymexazol for V_i read from calibration curve (ng)

7.3 Method recovery in plant

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Recovery of hymexazol from spinach fortified at 0.2 mg kg^{-1} is 86%.

8 Important points

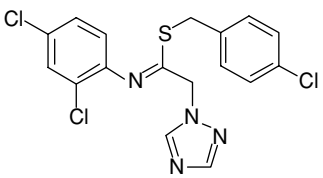
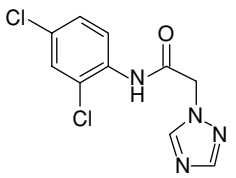
Hymexazol has such a high vapor pressure that the sample extract should not be concentrated to dryness without a keeper such as polyethylene glycol. After homogenization of plant materials, the extraction procedures should be carried out promptly without long-term storage.

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Imibenconazole

Materials to be analyzed	Apricots, apples, grapes, Japanese pears, melons, peaches, strawberries, tomatoes, watermelons and soil
Instrumentation	Gas-chromatographic determination for all materials

1 Introduction

	Imibenconazole	*Main metabolite (imibenconazole-debenzyl)*
Chemical name (IUPAC)	4-Chlorobenzyl *N*-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-yl) thioacetaimidate	2,4-Dichloro-2-(1*H*-1,2,4-triazol-1-yl)-acetanilide
Structural formula	 imibenconazole	 main metabolite
Empirical formula	$C_{17}H_{13}Cl_3N_4S$	$C_{10}H_8Cl_2N_4O$
Molar mass	411.7	271.1
Melting point	89.5–90 °C	178–179.5 °C
Vapor pressure	8.5×10^{-5} mPa at 25 °C	No data
Solubility	Water 1.7 mg L^{-1} at 20 °C Readily soluble in polar and nonpolar organic solvents	Water 87 mg L^{-1} at 25 °C
Other properties	Stable under weakly alkaline, unstable under acidic and strongly alkaline conditions	Stable under acidic and alkaline conditions
Use pattern	Imibenconazole is a triazole fungicide exhibiting direct cell membrane destruction as well as sterol biosynthesis inhibition for controlling major diseases of fruits, vegetables, cereals, ornamentals and turf.	

Handbook of Residue Analytical Methods for Agrochemicals.
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	Grape anthracnose and citrus scab caused by the genus *Elsinoe* are eradicated even after disease development by foliar application
Regulatory position	The residue definition is for both imibenconazole and its primary metabolite (imibenconazole-debenzyl)

2 Outline of method

Plant samples are homogenized with sodium hydrogencarbonate aqueous solution to prevent decomposition of the analytes during homogenization. Imibenconazole and its primary metabolite, imibenconazole-debenzyl, are extracted from plan materials and soil with methanol. After evaporation of methanol from the extracts, the residues are extracted with dichloromethane from the residual aqueous solution. The dichloromethane phase is cleaned up on Florisil and C_{18} columns. Imibenconazole and imibenconazole-debenzyl are determined by gas chromatography/nitrogen–phosphorus detection (GC/NPD).

3 Apparatus

Blender fitted with leak-proof glass jar and explosion-proof motor
Homogenizer, e.g. Polytron
Laboratory mechanical shaker
Conical beaker, 300-mL
Erlenmeyer flask, 200-mL, with ground stopper
Filter paper, 9.5-cm diameter, medium flow rate
Filtration flask, 1-L
Glass funnel with filter plate, 9.5-cm i.d.
Round-bottom flasks, 50-, 100- and 300-mL
Rotary vacuum evaporator, bath temperature 40 °C
Separatory funnels, 100- and 200-mL
Gas chromatograph equipped with a nitrogen–phosphorus detector

DropBooks

4 Reagents

Acetone, high purity
Acetonitrile, high purity
Dichloromethane, high purity
Ethyl acetate, high purity
n-Hexane, high purity
Imibenconazole standard solutions: 0.02, 0.1, 0.2, 0.4 and 1.0 μg mL^{-1} in acetone
Imibenconazole-debenzyl standard solutions: 0.02, 0.1, 0.2, 0.4 and 1.0 μg mL^{-1} in acetone
Methanol, high purity
Water, deionized or distilled
Sodium chloride, high purity

Sodium hydrogencarbonate aqueous solutions, 10% (w/v) and saturated solution
Sodium sulfate, anhydrous, high purity
C_{18} cartridge (Mega Bond Elut, 1-g/6-mL, Varian)
Florisil cartridge (Sep-Pak Plus, Waters)
Air, synthetic
Nitrogen, repurified
Hydrogen, repurified

5 Sampling and preparation

Plant samples (1000 g) are homogenized using a blender with 10% aqueous sodium hydrogencarbonate solution (400 mL). The pH of the homogenates must be adjusted to 6–8 with saturated aqueous sodium hydrogencarbonate solution in this step.

6 Procedure

6.1 Extraction

6.1.1 Plant samples

Weigh 35 g of the prepared homogenate (25 g for plant material) into a conical beaker. Homogenize the homogenate with 100 mL of methanol with a homogenizer for 2 min, immersing the beaker in an ice-bath. Filter the homogenate through a filter paper into a 300-mL round-bottom flask. Wash the filter cake with 80 mL of methanol, combine all the filtrates in the round-bottom flask and remove the methanol by rotary evaporation.

6.1.2 Soil

Shake 25 g (dry weight equivalent) of soil with 100 mL of methanol with a mechanical shaker for 30 min. Filter the mixture through a filter paper into a 300-mL round-bottom flask. Wash the filter cake with 80 mL of methanol, combine all the filtrates in the round-bottom flask and remove the methanol by rotary evaporation.

6.2 Cleanup

6.2.1 Liquid–liquid partition

Transfer the residue derived from Section 6.1.1 or 6.1.2 into a 200-mL separatory funnel with 80 mL of water and add 5 g of sodium chloride. Adjust the pH of the aqueous phase to 6–8 with saturated aqueous sodium hydrogencarbonate solution. Extract the aqueous phase successively with 50 and 30 mL of dichloromethane by shaking the funnel with a mechanical shaker for 5 min. Combine the dichloromethane extracts and dry with anhydrous sodium sulfate. Transfer the extracts into a 100-mL round-bottom flask and concentrate the extracts to near dryness by rotary evaporation. Dissolve the residue in 2 mL of n-hexane.

6.2.2 Florisil column chromatography

Pass the solution derived from Section 6.2.1 through a Florisil cartridge (conditioned prior to use successively with 5 mL of acetone and 10 mL of n-hexane), then elute interfering substances with 10 mL of n-hexane–acetone (19 : 1, v/v) and discard the eluate. Elute imibenconazole and imibenconazole-debenzyl with 25 mL of n-hexane–acetone (3 : 2, v/v). Collect the eluate in a 50-mL round-bottom flask and concentrate the eluate to dryness by rotary evaporation. Dissolve the residue in 5 mL of acetonitrile–water (3 : 17, v/v).

6.2.3 C_{18} column chromatography

Pass the solution derived from Section 6.2.2 through a C_{18} cartridge (conditioned prior to use successively with 5 mL of acetonitrile and 10 mL of water), then elute interfering substances with 15 mL of acetonitrile–water (3 : 17, v/v) and discard the eluate. Elute imibenconazole-debenzyl with 20 mL of acetonitrile–water (2 : 3, v/v) and collect the eluate in a 100-mL separatory funnel (imibenconazole-debenzyl fraction). Elute imibenconazole with 20 mL of acetonitrile–water (17 : 3, v/v) and collect the eluate in a 100-mL separatory funnel (imibenconazole fraction). Add 30 mL of 20% sodium chloride aqueous solution and 40 mL of ethyl acetate to each separatory funnel and shake the funnel with a mechanical shaker for 5 min. Collect the ethyl acetate extract, dry the extract with anhydrous sodium sulfate and transfer into a 100-mL round-bottom flask. Concentrate the ethyl acetate extract to near dryness by rotary evaporation and dry with a stream of nitrogen. Dissolve the residue of each fraction in acetone for gas chromatographic determination as in Section 6.3.

DropBooks

6.3 Gas-chromatographic determination

Inject an aliquot (V_i) of the solution derived from Section 6.2.3 (V_{End}) into the gas chromatograph.

Operating conditions

Gas chromatograph	Hewlett-Packard model 5890A
Column	Fused-silica capillary column, Rtx-200, 0.53-mm i.d., 15-m length, film thickness 1.5-μm (for imibenconazole) Fused-silica capillary column, OV-351, 0.53-mm i.d., 15-m length, film thickness 1.0-μm (for imibenconazole-debenzyl)
Column temperature	260 °C (for imibenconazole), 235 °C (for imibenconazole-debenzyl)
Injection port temperature	280 °C (for imibenconazole), 260 °C (for imibenconazole-debenzyl)
Detector	Nitrogen–phosphorus detector
Detector temperature	290 °C (for imibenconazole), 260 °C (for imibenconazole-debenzyl)

Gas flow rates	Helium carrier gas, 15 mL min^{-1} Hydrogen, 3.5 mL min^{-1} Air, 100 mL min^{-1}
Injection volume	2 μL
Retention times	4 min for imibenconazole, 5.9 min for imibenconazole-debenzyl

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Construct fresh calibration curves with imibenconazole and imibenconazole-debenzyl standard solutions for each set of analyses. Inject 2 μL of each standard solution (equivalent to 0.04–2 ng of imibenconazole and imibenconazole-debenzyl) into the gas chromatograph. Plot the peak areas obtained versus amount of imibenconazole and imibenconazole-debenzyl injected.

7.2 Recoveries, limit of detection and limit of determination

7.2.1 Plant samples

The recoveries from untreated control samples fortified with imibenconazole and imibenconazole-debenzyl at levels of 0.008–0.2 mg kg^{-1} ranged from 84 to 110% and from 88 to 115%, respectively. The limits of detection of imibenconazole and imibenconazole-debenzyl were 0.004 mg kg^{-1} for each compound. The limits of determination of imibenconazole and imibenconazole-debenzyl were 0.008 mg kg^{-1} for each compound.

7.2.2 Soil

The recoveries from blank soils fortified with imibenconazole and imibenconazole-debenzyl at levels of 0.04–0.4 mg kg^{-1} ranged from 86 to 96% and from 92 to 96%, respectively. The limits of detection of imibenconazole and imibenconazole-debenzyl were 0.01 mg kg^{-1} for each compound. The limits of determination of imibenconazole and imibenconazole-debenzyl were 0.04 mg kg^{-1} for each compound.

7.3 Calculation of residues

The residues R (mg kg^{-1}) of imibenconazole and the metabolite imibenconazole-debenzyl (calculated as imibenconazole by conversion factor) are calculated from the following equations:

for imibenconazole:

$$R = \frac{W_A \times V_{End}}{V_i \times G}$$

for imibenconazole-debenzyl:

$$R = \frac{W_A \times V_{End}}{V_i \times G} \times 1.519$$

where

G = sample weight (g)
V_{End} = terminal volume of sample solution from Section 6.2.3 (mL)
V_i = portion of volume V_{End} injected into the gas chromatograph (μL)
W_A = amount of imibenconazole or imibenconazole-debenzyl for V_i read from calibration curve (ng)
1.519 = factor for conversion of imibenconazole-debenzyl to imibenconazole

8 Important point

The pH of the sample must be adjusted to 6–8 in the homogenization step because imibenconazole is unstable under acidic and under strongly alkaline conditions.

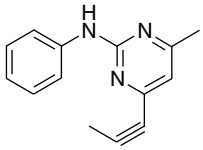
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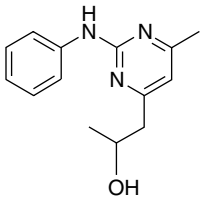
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Mepanipyrim

Materials to be analyzed	Plants (cucumber, tomato, eggplant, French bean, deep root leek, strawberry, grape, apple, pear, cherry, peach, Unshu orange, Chinese citron, watermelon, persimmon, lemon, kabosu lime, sudachi, small beans and kidney beans), soil and water
Instrumentation	Gas-chromatographic determination

1 Introduction

Chemical name (IUPAC)	*N*-(4-Methyl-6-prop-1-ynylpyrimidin-2-yl)aniline
Structural formula	
Empirical formula	$C_{14}H_{13}N_3$
Molar mass	223.3
Melting point	132.8 °C
Vapor pressure	2.32×10^{-2} mPa (20 °C)
Solubility	Water 3.10 mg L^{-1} (20 °C) Acetone 139, methanol 15.4, n-hexane 2.06 g L^{-1} (20 °C)
Stability	Stable in water (DT_{50} >1 yr at pH 4–9) Stable to heat (no change over 14 days at 55 °C) Stable to light in water (DT_{50} 12.9 days)
Use pattern	Mepanipyrim is a nonsystemic fungicide to control gray mould on vines, tomatoes, etc., and scabs on pome fruits.
Regulatory position	The residue definition include mepanipyrim and its hydroxylated metabolite 1-(2-anilino-6-methylpyrimidin-4-ynyl)-2-propanol (abbreviated as propanol form)



Propanol form

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2 Outline of method

Mepanipyrim in crop samples is recovered by acetone solvent extraction. The acetone is evaporated under reduced pressure and the residual aqueous extract is hydrolyzed with enzyme (β-glucosidases) to release hydroxylated metabolite(s). After enzyme treatment, mepanipyrim and the propanol form metabolite are extracted with dichloromethane, purified by silica gel column chromatography and quantified by gas chromatography/nitrogen–phosphorus detection (GC/NPD).

The soil samples are extracted by refluxing with a mixture of acetone and water. Mepanipyrim in the extract is purified by silica gel column chromatography and determined by GC/NPD.

The water samples are extracted by solid-phase extraction (SPE) and analyzed by GC/NPD.

3 Equipment

Crusher (sample mill)
Blender (kitchen type)
Round-bottom flasks: 200-, 300- and 500-mL
Buchner funnel: 10-cm i.d.
Separatory funnel: 200-mL
Condenser
Centrifuge tube with ground stopper: 100-mL
Glass chromatography column: 400 × 15-mm i.d. with a stopcock
Column preparation: A silica gel column is prepared by packing a slurry of silica gel (10 g) in n-hexane–ethyl acetate (9 : 1, v/v) solvent mixture into a glass chromatography column. About a 1-cm layer of anhydrous sodium sulfate is placed above and below the silica gel bed
SPE cartridge column: Mega Bond Elut C_{18}, 10-g/60-mL (Varian). The SPE cartridge column is rinsed with 100 mL of methanol and 100 mL of distilled water
Rotary vacuum evaporator, 40 °C bath temperature
Water-bath, electrically heated, temperature 80 °C
Mechanical shaker (universal shaker)
Shaking incubator

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4 Reagents

Acetone, dichloromethane, ethyl acetate and n-hexane, pesticide residue analysis grade
Distilled water: high-performance liquid chromatography grade
Acetate buffer: 0.2 M, pH 5.0
Sodium chloride: special grade
Anhydrous sodium sulfate: special grade
β-Glucosidases: from almonds, EC 3.2.1.21 (Sigma)
Cellulases: from *Aspergillus niger*, EC 3.2.1.4 (Sigma)

Silica gel: Wakogel C-200 (Wako Pure Chemical Industries, Ltd)
pH test paper
Mepanipyrim: analytical standard material (Ihara Chemical Industry Co., Ltd)
Mepanipyrim propanol form: 1-(2-anilino-6-methylpyrimidin-4-ynyl)-2-propanol analytical standard material (Ihara Chemical Industry Co., Ltd)
Standard solutions for gas chromatography: 0.2 and 1.0 $\mu g\,mL^{-1}$ acetone

5 Sample preparation

For fruits and vegetables, mince and homogenize 1 kg of the sample with a mixer together with an appropriate amount of water, if necessary, and weigh 50 g of the sample. For small fruit samples such as citron, kabosu lime and rind, weigh 20–25 g of the sample. For beans, after grinding the sample, weigh 20 g of the sample and add 40 mL of distilled water to swell the sample for 2 h.

6 Procedure

6.1 Extraction

6.1.1 Plant material

Add 100 mL of acetone to the sample in a 300-mL round-bottom flask and shake for 1 h. Filter the mixture through a filter paper on a Buchner funnel into a 500-mL round-bottom flask by suction. Wash the residue and the flask with 70 mL of acetone and filter the washings in the same manner. Combine the filtrate and concentrate it under reduced pressure to 30 mL (20 mL for citron). Transfer the concentrate into a 100-mL centrifuge tube. Wash the flask with 30 mL of 0.2 M acetate buffer (pH 5.0) and combine the washings into the centrifuge tube. Add 150 U of β-glucosidases and 100 U of cellulases and shake the tube at 37 °C for 18 h (60 rpm). After the treatment with enzyme, adjust the solution to pH 8–9 with 2 M sodium hydroxide solution. Transfer the solution into a 300-mL separatory funnel and extract three times with 70 mL of dichloromethane. Dry the dichloromethane extract through about 50 g of anhydrous sodium sulfate on a glass funnel and collect it in a 300-mL round-bottom flask. Evaporate the solvent under reduced pressure. Dissolve the residue in a small volume of n-hexane–ethyl acetate (9 : 1, v/v) mixture.

6.1.2 Soil

Weigh 40 g (dry weight basis) of the soil sample into a 500-mL round-bottom flask and add 200 mL of acetone–water (3 : 1, v/v) mixture. Attach a condenser to the flask and perform reflux extraction at 80 °C for 1 h. Filter the extract through a filter paper on a Buchner funnel with suction into a 500-mL round-bottom flask. Rinse the residue on the funnel with 130 mL of acetone, and filter in the same manner. Combine and concentrate the extract under reduced pressure to 50 mL. Transfer the concentrate into a 200-mL separatory funnel with 10 mL of saturated aqueous sodium chloride

solution. Extract twice with 70 mL of dichloromethane. Dry the dichloromethane extract through 50 g of anhydrous sodium sulfate on a glass funnel and collect it in a 300-mL round-bottom flask. Concentrate the extract to dryness under reduced pressure. Dissolve the residue in an appropriate volume of n-hexane–ethyl acetate (9 : 1, v/v) mixture.

6.1.3 Water

Apply 500 mL of the water sample in an SPE tube (Mega Bond Elut C_{18}). Rinse the SPE tube with 30 mL of distilled water and 40 mL of n-hexane. Elute the SPE tube with 50 mL of n-hexane–ethyl acetate (4 : 1, v/v) mixture.

6.2 Cleanup

6.2.1 Plant material

Prepare a silica gel column as mentioned in Section 3. Transfer the solution derived from Section 6.1.1 to the column. Wash the flask with a small volume of n-hexane–ethyl acetate (9 : 1, v/v) mixture and elute with the same solvent mixture. Discard the first 50 mL of eluate and collect the next 110 mL of eluate in a 200-mL round-bottom flask (mepanipyrim fraction). Then, elute the column with 100 mL of n-hexane–ethyl acetate (1 : 1, v/v) mixture and collect the eluate (propanol form the metabolite fraction). Evaporate the solvents under reduced pressure and dissolve the residue in an appropriate volume of acetone for analysis.

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6.2.2 Soil

Prepare a silica gel column as mentioned in Section 3. Transfer the solution derived from Section 6.1.2 to the column. Wash the flask with a small volume of n-hexane–ethyl acetate (9 : 1, v/v) mixture and elute with the same solvent mixture. Discard the first 40 mL of eluate and collect the next 100 mL of eluate in a 200-mL round-bottom flask. Concentrate the eluate to dryness under reduced pressure and dissolve the residue in an appropriate volume of acetone for analysis.

6.2.3 Water

Concentrate the eluate from the SPE tube (Section 6.1.3) to 1 mL under reduced pressure and evaporate the residual solvent under a gentle stream of air. Dissolve the residue in an appropriate volume of acetone for analysis.

6.3 Gas-chromatographic determination

Inject an aliquot (V_i) of the solution derived from Section 6.2 (V_{End}) into the gas chromatograph.

Operating conditions

Gas chromatograph	Hewlett-Packard Model 5890
Column	FFAP Megabore column, 0.53-mm i.d., 10-m length, 1.0-μm film thickness (Hewlett-Packard)
Column temperature	215 °C (mepanipyrim), 220 °C (propanol form)
Injection port temperature	240 °C
Detector	Nitrogen–phosphorus flame ionization detector
Detector temperature	250 °C
Gas flow rates	Helium carrier gas, 20 mL min^{-1} Hydrogen, 4 mL min^{-1} Air, 100 mL min^{-1}
Attenuation	2
Injection volume	1–4 μL
Retention time	2.7 min (mepanipyrim), 2.9 min (propanol form)
Minimum detectable amount	0.2 ng

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Construct a fresh calibration curve with mepanipyrim standard solutions for each set of analysis. Using log–log paper, plot the peak heights in millimeters against the injected amount of mepanipyrim in nanograms. Measure the peak heights of mepanipyrim on a chromatogram of a sample extract and quantify by comparing the peak height with the calibration curve. Prepare the calibration curve similarly as for the propanol form, and conduct quantitation.

7.2 Recoveries and limit of detection

The recoveries and the limit of detection from untreated control crops fortified with mepanipyrim are given in Table 1.

The recoveries from control soils fortified with mepanipyrim at 0.2 mg kg^{-1} ranged from 89 to 100% (RSD <4.6%). The limit of detection was 0.005 mg kg^{-1}.

The recoveries from control water fortified with mepanipyrim at 0.05 and 0.5 μg kg^{-1} were 83% (RSD 3.2%) and 89% (RSD 2.8%), respectively. The limit of determination was 0.05 μg kg^{-1}.

The recoveries of the propanol form from the control crops and soil were 81–96% (at 0.2 mg kg^{-1}) and 80–94% (at 0.5 mg kg^{-1}), respectively.

Table 1 Recoveries and limits of detection

Crop		Fortified concentration (mg kg^{-1})	Mean recovery (%)	RSD (%)[a]	Limit of detection (mg kg^{-1})
Vegetables	Cucumber, tomato, eggplant, French beans, deep-root leek	0.1	82–102	<4.3	0.002
Fruits	Strawberry, grape, apple, pear, cherry, peach, unshu orange, Chinese citrus, watermelon[b], persimmon[b], lemon[b], kabosu lime[b], sudachi[b]	0.1	80–98	<6.2	0.002
Rind	Peach, unshu orange, Chinese citrus	0.2	85–95	<3.8	0.004

[a] RSD = relative standard deviation.
[b] Fortified concentration is 0.25 mg kg^{-1}.

7.3 Calculation of residues

The residue R, expressed in mg kg^{-1} mepanipyrim, is calculated from the following equation:

$$R = (W_A \times V_{End})/(V_i \times G)$$

DropBooks

where

G = sample weight (g)
V_{End} = terminal volume of sample solution from Section 6.2 (mL)
V_i = portion of volume of V_{End} injected into gas chromatograph (μL)
W_A = amount of mepanipyrim for V_i read from calibration curve (ng)

8 Important points

8.1 Analysis of plant metabolites

Since enzyme hydrolysis is used to analyze the propanol conjugate, the primary metabolite of mepanipyrim, this enzyme hydrolysis step can be omitted if only mepanipyrim is analyzed. The propanol form is mostly present as multiple glucosides in plants. Therefore, the amount of residue is determined by hydrolyzing conjugates to aglycone and combining with the free form. Since the propanol form is unstable to acid, enzymatic treatment to release the conjugate is used. When the conditions of enzymatic treatment were examined on a grape sample treated with mepanipyrim, hydrolysis was insufficient with a single enzyme (release rate: cellulases > pectinases $\gg$ β-glucosidases). If two enzymes were used concurrently, the amount released increased. When mepanipyrim-treated grape or strawberry samples were hydrolyzed with a mixture of cellulases and β-glucosidases for 18 h, the

hydrolysis reaction was optimized with 100 U of cellulases plus 150 U of β-glucosidases.

8.2 Extraction rate from soil

The extraction rate of mepanipyrim with refluxing was higher than that with shaking (30 min) and sonication (Ultrasonic, 600 W, 28 kHz, 30 min). For the solvent system, acetone and acetonitrile showed almost similar extraction efficiencies. Methanol was found to be a less effective extraction solvent. Mepanipyrim was unstable in the acidic solution and alkaline solution under reflux conditions at 80 °C. The extraction rate of mepanipyrim under these conditions decreased to about 50% and 20%, respectively. Therefore, neutral solution was used as the extraction solvent in this method.

Further reading

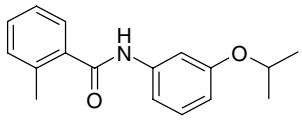
M. Ikeda, Y. Asano, Y. Maeda, and Y. Yusa, *J. Pestic. Sci.,* **23**, 1 (1998).
M. Ikeda, Y. Asano, Y. Maeda, and Y. Yusa, *J. Pestic. Sci.,* **23**, 9 (1998).
M. Ikeda, K. Mizumo, T. Oishi, Y. Nakahira, S. Oishi, Y. Asano, and K. Ishikawa, 'Analytical methods of mepanipyrim and its metabolites in crops and soils,' in "Abstracts of the 17th Annual Meeting of the Pesticide Science Society of Japan," p. 146 (1992).
Y. Nakahira, O. Kimura, H. Aoshima, M. Ikeda, Y. Asano, and Y. Yusa, 'Analysis of the residue of a new fungicide, mepanipyrim, and its metabolites in crops,' in "Abstracts of the 18th Annual Meeting of the Pesticide Residue Analysis Society of Japan, pp. 1–10 (1994) (in Japanese).

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Mepronil

Materials to be analyzed	Rice (rice grain, rice straw), leek, lettuce, sugar beet (root), sugar beet (leaf), kidney beans, string beans and soil
Instrumentation	Gas-chromatographic determination

1 Introduction

Chemical name (IUPAC)	3′-Isopropoxy-*o*-toluanilide
Structural formula	
CAS No.	55814-41-0
Empirical formula	$C_{17}H_{19}NO_2$
Molar mass	269.3
Melting point	92–93 °C
Vapor pressure	0.056 mPa (25 °C)
Solubility	Water 12.7 g L^{-1} (25 °C) Methanol >500, acetone >500, acetonitrile 314, benzene 282, n-hexane 1.1 g L^{-1} (20 °C)
Stability	Stable in neutral, acidic and weakly alkaline conditions. Hydrolyzed in highly alkaline conditions. Stable to light and heat
Use pattern	Mepronil is a systemic fungicide to control diseases caused by *Basidiomycetes* in cereals, rice, potatoes, vegetables, etc.
Regulatory position	The residue definition is for the parent, mepronil only.

DropBooks

2 Outline of method

Mepronil in plant materials is extracted with aqueous acetone. Rice straw sample is extracted with aqueous methanol. Soil samples are refluxed with alkaline methanol. After filtration, the solvent is removed by evaporation under reduced pressure and

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the residue is extracted with n-hexane. The extract is purified by alumina column chromatography. Mepronil is determined by gas chromatography with nitrogen–phosphorus detection (NPD).

3 Apparatus

Mill (coffee-mill type)
Grinder (cutting mills, Willey type)
Food mixer
Homogenizer (Polytron mixer)
Ultrasonic cleaner, UC-6100, 600 W, 28 kH (Sharp)
Buchner funnel, 11-cm i.d.
Round-bottom flasks, 1-L, 500-mL and 300-mL with ground joints
Erlenmeyer flask, 500-mL
Rotary vacuum evaporator, 40 °C bath temperature
Separatory funnel, 300-mL
Funnel, 10-cm diameter
Glass chromatography column, 15-mm i.d. × 400 mm with a stopcock
Alumina column: Place a cotton wool plug and then add anhydrous sodium sulfate in a layer 1-cm thick at the bottom of a glass chromatography column. Weigh 20 g of aluminum oxide 90 (Merck) and pour it into the tube with n-hexane–ethyl acetate (9 : 1, v/v). Rinse the alumina column with the same solvent system and place anhydrous sodium sulfate in a layer 1-cm thick on the top of the column
Gas chromatograph, equipped with a nitrogen–phosphorus detector
Microsyringe, 10-μL
Volumetric flasks, 5- and 10-mL

4 Reagents

Distilled water, high-performance liquid chromatography grade
Acetone, pesticide residue analysis grade
Ethyl acetate, pesticide residue analysis grade
n-Hexane, pesticide residue analysis grade
Methanol, pesticide residue analysis grade
Mepronil standard solution: A 100-mg amount of mepronil is dissolved in 100 mL of acetone to prepare a 1000-mg L^{-1} acetone solution. This solution is diluted with acetone to prepare 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 10.0 mg L^{-1} acetone standard solutions.
Anhydrous sodium sulfate, special grade
Sodium hydroxide (Na OH), special grade
Hydrochloric acid (HCl), special grade
Aluminum oxide 90, neutral, activity grade II–III (Merck)
Cotton wool
Filter aid, Celite 545 (Johns-Manville Products Corporation)

5 Sampling and sample preparation

The soil sample is prepared by manually removing stones and plant materials and passing it through a 5-mm sieve.

6 Procedure

6.1 Extraction

6.1.1 Rice straw

Weigh 10 g of the sample into a 500-mL Erlenmeyer flask and add 120 mL of water–methanol (1 : 9, v/v). Reflux the mixture at 70 °C for 30 min. Filter the extract through a filter paper overlaid with 20 g of Celite in a Buchner funnel into a 1-L round-bottom flask with suction. Rinse the flask and the filter cake with 100 mL of water–methanol (1 : 9, v/v). Combine the filtrates and concentrate to approximately 15 mL under reduced pressure at 40 °C. Then the residue is processed as described in Section 6.2.1.

6.1.2 Rice grain

Weigh 10 g of the sample into a 500-mL Erlenmeyer flask and add 120 mL of water–acetone (1 : 9, v/v). Sonicate the mixture for 30 min. Carry out the subsequent procedures in a same manner as for rice straw.

DropBooks

6.1.3 Plant samples except for rice grain and rice straw

Weigh 50 g of the sample into a 500-mL Erlenmeyer flask. Homogenize the sample with 150 mL of water–acetone (1 : 5, v/v) for 1 min using a Polytron. Carry out the subsequent procedures in a same manner as for rice straw.

6.1.4 Soil

Weigh 50 g (dry soil) of the sample into a 500-mL round-bottom flask, add 160 mL of 6 M NaOH–methanol (1 : 3, v/v) and reflux the mixture at 70 °C for 1 h. Filter the extract through a filter paper overlaid with 20 g of Celite in a Buchner funnel into a 1-L round-bottom flask with suction. Rinse the flask and the filter cake twice with 30 mL of methanol. Combine the filtrates and concentrate to approximately 40 mL under reduced pressure. Neutralize the residue with 6 N HCl. Then the residue is processed as described in Section 6.2.1.

6.2 Cleanup

6.2.1 Liquid–liquid partition

Into a 300-mL separatory funnel transfer the residue prepared in Section 6.1.1, 6.1.2 or 6.1.3 with 100 mL of distilled water and add 10 mL of saturated sodium chloride aqueous solution. Extract the mixture twice with 50 mL of n-hexane. Combine the

extracts and filter into a 500-mL round-bottom flask through 60 g of anhydrous sodium sulfate supported by a plug of cotton wool in a funnel. Concentrate the filtrate to dryness under reduced pressure.

6.2.2 Column chromatography

Prepare an alumina column as described in Section 3. Dissolve the residue prepared in Section 6.2.1 in 3 mL of n-hexane–ethyl acetate (10 : 1, v/v) and transfer the solution to the column. Rinse the flask twice with 5 mL of the same solvent system and transfer these solutions into the column. Allow the solution to percolate through the column and discard the eluate. Add 160 mL of n-hexane–ethyl acetate (10 : 1, v/v) to the column. Discard the first 40 mL of eluate and collect the second 120 mL of eluate in a 300-mL round-bottom flask. Evaporate the eluate to dryness under reduced pressure.

6.3 Gas-chromatographic determination

Dissolve the residue prepared in Section 6.2.3 in acetone. Transfer the solution to a volumetric flask and make up to a given volume, e.g. 5 mL (V_{End}) with acetone. Inject an aliquot of the solution (V_i) into the gas chromatograph.

Operating conditions

Gas chromatograph	HP 5710A (Hewlett-Packard)
Column	Glass, 30 cm × 2.0-mm i.d., packed with 1% FFAP on Gaschrom-Q, 60–80 mesh
Column temperature	260 °C
Injection temperature	300 °C
Detector	Nitrogen–phosphorus detector
Detector temperature	300 °C
Gas flow rates	Helium carrier gas, 20 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 100 mL min^{-1}
Chart speed	0.5 cm min^{-1}
Injection volume	1–5 μL
Retention time for mepronil	2.0 min

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Prepare a calibration curve by injecting the standard solutions, equivalent to 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 10.0 ng of mepronil, into the gas chromatograph. Measure the heights of the peaks obtained. Plot the peak heights in millimeters against the injected amounts of mepronil in nanograms. Measure the peak height of mepronil on the chromatogram of the sample solution and quantify mepronil by comparing the peak height with the calibration curve.

7.2 Recoveries, limit of detection and limit of determination

Untreated control samples were fortified with mepronil. The fortification levels were 0.05–0.25 mg kg^{-1} for plant materials and 0.005–0.05 mg kg^{-1} for soil. The following recoveries were obtained: 93–95% from rice grain; 93–99% from rice straw; 86–96% from grape; 99–103% from leek; 90–110% from lettuce; 96–106% from sugar beet (root); 92–100% from sugar beet (leaf); 91–96% from kidney beans; 96–100% from string beans; and 86–98% from soil. The limit of detection is 0.005 mg kg^{-1} for plant samples, except for rice straw and soil materials, and 0.01 mg kg^{-1} for rice straw.

7.3 Calculation of residues

The residue R, expressed in mg kg^{-1} mepronil, is calculated from the following equation:

$$R = (W_A \times V_{End})/(V_i \times G)$$

where

G = sample weight (g)
V_{End} = final volume of sample solution from Section 6.3 (mL)
V_i = portion of volume V_{End} injected into the gas chromatograph (μL)
W_A = amount of mepronil for V_i read from the calibration curve (ng)

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8 Important points

Instead of the packed column mentioned in Section 6.3, a megabore column may also be used for gas-chromatographic determination. The operating conditions are as follows:

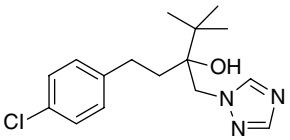
Gas chromatograph	HP 5890A (Hewlett-Packard)
Column	DB-1, 1.0-μm thickness, 15 m × 0.53-mm i.d. (J&W Scientific)
Column temperature	210 °C
Injection temperature	240 °C
Detector	Nitrogen–phosphorus detector
Detector temperature	250 °C
Gas flow rates	Helium carrier gas, 20 mL min^{-1} Helium make-up gas, 10 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 100 mL min^{-1}
Retention time	3.0 min

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Tebuconazole

Materials to be analyzed	Field and sweet corn, soybeans, tomatoes, soil, and water
Instrumentation	Liquid chromatography/tandem mass spectrometry (LC/MS/MS) for plant material and soil, gas chromatography (GC) for water

1 Introduction

Chemical name (IUPAC)	α-[2-(4-Chlorophenyl)ethyl]-α-(1,1-dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol
CAS No.	107534-96-3
Structural formula	
Physical form	Solid
Color	White to off-white
Empirical formula	$C_{16}H_{22}ClN_3O$
Molar mass	307.8
Melting point	102–105 °C
Boiling point	140 °C at 3.1×10^{-2} mmHg
Vapor pressure	9.8×10^{-9} mmHg at 20 °C
Solubility	Water: 32 mg L^{-1} at 20 °C Readily soluble in polar organic solvents
Stability	Stable in pH 4–9 aqueous solution Stable in most organic solvents
Use pattern	Tebuconazole is a broad-spectrum triazole fungicide which provides protective and, in some cases, curative activity on a wide variety of crops including cereal grains, peanuts, grapes, bananas, pome fruit, stone fruit, nuts, and vegetables
Regulatory position	The residue definition consists of tebuconazole alone for crops, soil, and water

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2 Outline of method

Macerated plant material is homogenized with acetone–water (3 : 1, v/v) and vacuum filtered, and the filtrate is adjusted to constant volume. A portion of the filtrate is further filtered through a syringe filter and diluted 1 : 1 with an isotopically labeled internal standard solution for analysis by electrospray LC/MS/MS.

Soil samples are extracted with methanol–water (7 : 3, v/v) using a Soxtec extractor. After addition of an isotopically labeled internal standard (IS) and dilution to 50 mL, the extracts are analyzed by electrospray LC/MS/MS.

Water samples are directly partitioned with dichloromethane (DCM). The DCM extract is then rotary evaporated and driven to dryness with a stream of nitrogen. The dry residue is dissolved in acetone and analyzed by gas chromatography/nitrogen–phosphorus detection (GC-NPD).

3 Apparatus

Assorted laboratory supplies including, but not limited to, the following.
Beakers, tall-form, stainless steel, 300-mL, 1-L
Buchner funnels, 9-cm
Capillary tubes, 100-μL, accurate to ±1%, with control syringe
Cylinders, graduated, 100-mL
Cylinders, graduated mixing with ground-glass joints and stoppers, 50-, 250-mL
Flasks, round-bottom with 24/40 ground-glass joint, 500-mL
Flasks, volumetric, Class A, various sizes
Funnels, glass
Pipets, volumetric, Class A, various sizes
Separatory funnels, 1-L
Spatulas, stainless steel
Balances: top-loader electronic, accurate to 0.0001 g and to 0.01 g
Columns, high-performance liquid chromatography (HPLC): Luna C8(2), 5-μm, 100 × 4.6-mm i.d. (Phenomenex, Torrance, CA, USA) or equivalent (30 °C); Prodigy 5 ODS-2, 5-μm, 125 mm × 4.6-mm i.d. (Phenomenex) or equivalent (ambient temperature)
Column, (GC): HP-1, 30 m × 0.32-mm i.d. 0.25-μm film thickness (Agilent Technologies) or equivalent.
Degasser, HPLC mobile phase
Evaporator, rotary vacuum, water-bath set at 35 °C
Disk mill
Hammer mill
Vertical batch processor
Gas chromatograph, Model 3400 (Varian, San Jose, CA, USA) equipped with split/splitless injector and a nitrogen–phosphorus detector (GC/NPD) or equivalent
Heater, HPLC column
Mass spectrometer, TSQ 7000 with API electrospray source and gradient HPLC input (Finnigan MAT, San Jose, CA, USA) or equivalent
Soxtec extraction unit, HT 1043 with extraction cups (Tecator, Hoganas, Sweden) or equivalent

DropBooks

Tissumizer, Model SDT 1810 S1 with S25N probe (Tekmar, Cincinnati, OH, USA) or equivalent

4 Reagents/supplies and reference standards

4.1 Reagents/supplies

Ammonium acetate, HPLC grade (Fisher Scientific, Fair Lawn, NJ) or equivalent
Bags, sample storage, plastic
Bottles: round, flint glass with polymer-lined lids, 250-mL; square, flint glass with polymer-lined lids, 0.5-oz
Dry-ice, pelletized
Extraction thimbles, Soxtec (Tecator) or equivalent
Filter aid, Celite 545 (Fisher Scientific) or equivalent
Filter cartridges, Acrodisc nylon, 25-mm (Gelman Sciences, Ann Arbor, MI, USA) or equivalent
Filter paper, GF/A, 9-cm
Formic acid, 88% aqueous solution (J.T. Baker, Phillipsburg, NJ, USA) or equivalent
Glass wool
Sodium sulfate, anhydrous, ACS grade (Fisher Scientific) or equivalent
Solvents: acetonitrile (ACN), acetone, dichloromethane (DCM), methanol (MeOH) and water (pesticide or HPLC grade)
Gas, compressed (breathing grade), air
Gases, compressed (purity ≥99%), argon, helium, hydrogen, nitrogen
Syringes, LuerLok, single-use, 10-mL
Vials: autosampler, clear glass with Teflon-lined septum caps, 1.8-mL; sample storage, clear glass with Teflon-lined caps, 12-mL

4.2 Reference materials

Tebuconazole (provided by Bayer), α-[2-(4-chlorophenyl)ethyl]-α-(1,1-dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol. Molar mass 307.8, $(M+H)^+$ ion observed at approximately m/z 308.1 [liquid chromatography/mass spectrometry (LC/MS)]
Tebuconazole-*triazole-1,2,4-*$^{15}N_3$ (provided in acetonitrile solution by Bayer), [$^{15}N_3$]tebuconazole stable-isotope internal standard, α-[2-(4-chlorophenyl)ethyl]-α-(1,1-dimethylethyl)-1*H*-$^{15}N_3$-1,2,4-triazole-1-ethanol. Molar mass 310.8, $(M+H)^+$ ion observed at approximately m/z 311.1 (LC/MS)

5 Sampling and preparation

Plant material should be added to a disk mill (grain or seed matrices) or a vertical batch processor (all other matrices). Add an equal portion of pelletized dry-ice to the sample (vertical processor only). Macerate the plant sample (or sample + dry-ice) until a homogeneous mixture is obtained. Soil samples should be well mixed or

homogenized, preferably using a hammer mill or other homogenization device. Store the homogenized mixture in doubled plastic bags at $-20 \pm 5\,^{\circ}C$. Samples containing dry-ice should be allowed to stand in the open bag in a freezer overnight to allow the dry-ice to sublime prior to sealing the bag. No specific sample preparation or processing is required for water samples

6 Procedures

6.1 Extraction

6.1.1 Plant material

Weigh 20.0 g of frozen homogenized sample into a 300-mL tall-form beaker. Begin recovery samples at this point by fortifying the control tissue matrix samples. Add 200 mL of acetone–water (3 : 1, v/v), and blend this mixture with a Tissumizer fitted with an S25N mixing probe for at least 3 min. Add approximately 10 g of filter aid to the sample in the beaker and swirl or stir the mixture well. Transfer the contents of the beaker into a 9-cm Buchner funnel containing GF/A paper, and vacuum filter the extract into a 250-mL graduated mixing cylinder. Rinse the extraction beaker and filter cake with 40 mL of acetone–water (3 : 1, v/v) while vacuum filtering and combining this rinse with the initial filtrate. Maintain vacuum until the filter cake is dry and discard the filter cake. Use additional acetone–water (3 : 1, v/v) to adjust the filtrate volume to 250 mL and mix the contents of the cylinder well.

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6.1.2 Soil

Weigh 20 g of sample into a Soxtec extraction thimble. Add 50 mL of methanol–water (7 : 3, v/v) to an aluminum Soxtec cup. Add an aliquot of internal standard solution into the cup. Place the Soxtec thimble and cup into the Soxtec unit. Reflux samples on the Soxtec apparatus using the 'Boiling' mode for 60 min at $140\,^{\circ}C$. Rinse samples using the 'Rinse' mode for an additional 30 min. Quantitatively transfer the extract to a 50-mL graduated mixing cylinder or volumetric flask. Rinse the Soxtec cup with a few milliliters of methanol–water (7 : 3, v/v), and transfer the rinsate to the graduated cylinder. Dilute the final extract to 50 mL using methanol–water (7 : 3, v/v).

6.1.3 Water

Transfer 500 mL of water into a 1-L separatory funnel. Extract the sample with three portions of 75 mL of DCM and combine all DCM.

6.2 Cleanup

6.2.1 Plant material

Attach a 25-mm Acrodisc nylon filter cartridge to the LuerLok fitting of a 10-mL disposable syringe, and transfer approximately 5 mL of filtrate to the syringe barrel.

Force the solution through the filter cartridge into a clean glass vial with a Teflon-lined lid for storage. Just prior to analysis, this filtered extract is diluted 1 : 1 (v/v) with internal standard solution. The combination of extract with internal standard solution may be made directly into the instrument autosampler vial.

6.2.2 Soil

Attach a 25-mm Acrodisc nylon filter cartridge to the LuerLok fitting of a 10-mL disposable syringe, and transfer approximately 1.5 mL of extract to the syringe barrel. Force the solution through the filter cartridge directly into a clean instrument autosampler vial.

6.2.3 Water

Pass the extract through approximately 5 g of anhydrous sodium sulfate held in a glass funnel into a 500-mL boiling flask. Concentrate the extract to 2–5 mL by vacuum rotary evaporation, transfer the solvent into a 0.5-oz square glass bottle and take the residue to dryness under a stream of nitrogen. Dissolve the residue in a 0.5 mL or larger volume of acetone for analysis.

6.3 Determination

6.3.1 Plant material and soil

Instrument	Finnigan MAT TSQ 7000 with atmospheric pressure ionization (API) electrospray interface and gradient HPLC, or equivalent
MS mode	Positive ion selected reaction monitoring (+SRM) Instrument parameters (sheath and auxiliary gas flows, spray voltage, capillary temperature, collision cell gas flow and offset, etc.) should be optimized while infusing a standard of tebuconazole prior to the first attempt at analysis. Optimization should be performed at an HPLC flow rate and composition simulating those present during elution of tebuconazole using each HPLC condition set employed
Ions detected	Tebuconazole m/z 70, product ion of m/z 308.1 [$^{15}N_3$]Tebuconazole m/z 73, product ion of m/z 311.1

HPLC conditions

<u>*Plant material extracts*</u>:

Column	Phenomenex Luna C8(2), 5-μm, 100 × 4.6-mm i.d., or equivalent
Column temperature	30 °C
Flow rate	1.5 mL min^{-1}
Post-column split	6 : 1 (14.3% to detector)
Injection volume	200 μL
Mobile phase A	0.1% (v/v) formic acid in water
Mobile phase B	Acetonitrile

Gradient	60% A (40% B) from 0 to 1 min, 35% A (65% B) at 7 min, 0% A (100% B) at 9 min, hold until 12 min, 60% A (40% B) at 13 min and hold until 18 min
Retention time	5.5 min (approximate)
Soil extracts:	
Column	Phenomenex Prodigy 5 ODS-2, 125 × 4.6-mm i.d., or equivalent
Column temperature	Ambient
Flow rate	0.8 mL min^{-1}
Post-column split	4 : 1 (20% to detector)
Injection volume	50 μL
Mobile phase A	5 mM NH_4OAc in water
Mobile phase B	Methanol
Gradient	80% A (20% B) from 0 to 1 min, 10% A (90% B) at 6 min, hold until 11 min, 80% A (20% B) at 11.5 min and hold until 16.5 min
Retention time	8.5 min (approximate)

6.3.2 Water

Instrument	Varian 3400 gas chromatograph with split/splitless injector and nitrogen–phosphorus detector (GC/NPD)
Column	HP-1 capillary column, 30 m × 0.32-mm i.d., 0.25-μm film thickness
Injection port	Mode splitless, temperature 250 °C
Detector temperature	300 °C
Gases/flow rates	Helium at 2 mL min^{-1} (carrier gas) Hydrogen at 4–5 mL min^{-1} Air at 170 mL min^{-1} Nitrogen at 26 mL min^{-1} (makeup gas)
Injection volume	5 μL
Column program	180 °C from 0 to 1 min, 10 °C min^{-1} to 230 °C, held for 15 min
Retention time	15 min (approximate)

DropBooks

7 Evaluation

7.1 Method

7.1.1 Plant material

Combine a 0.5-mL aliquot of the final sample extract or a 0.5-mL aliquot of the tebuconazole portion [0.004 mg L^{-1} in acetone–water (3 : 1, v/v)] of an external calibration standard, with a 0.5-mL aliquot of the [$^{15}N_3$]tebuconazole internal standard (IS) portion [0.004 mg L^{-1} in acetonitrile–water (1 : 4, v/v)] of the external calibration standard. Combination may be made using other volumes as long as the solutions are

combined 1 : 1 (v/v). Combination of the solutions is to be performed directly into the instrument autosampler vial. The external calibration standard solution combination is utilized to bracket a series of 1–10 injections of various sample extract–IS solution combinations. Calculation of final sample extract tebuconazole concentration is made by comparison of the response ratio (tebuconazole/[$^{15}N_3$]tebuconazole) in the sample with the averaged response ratios found in the external calibration solution injections that bracket the sample.

Instrumental response ratios (tebuconazole/[$^{15}N_3$]tebuconazole) versus concentration of tebuconazole present should be proven in solvent and each matrix analyzed up to the highest undiluted final sample extract concentration expected. Once proven linear, final sample extract residues found to lie above the range of linearity are to be diluted prior to addition of IS solution and re-analyzed.

7.1.2 Soil

Since IS solution is added to the sample at a point just after extraction, no addition of IS to the final sample extract is required. The concentration (mg kg^{-1}) of tebuconazole is calculated by applying the peak-area ratio (tebuconazole/[$^{15}N_3$] tebuconazole) with a calibration curve analyzed throughout the analytical sequence. All sample extract response ratios must fall within the limits of the calibration curve injected over the course of sample analyses. Samples showing a response ratio above the limit of the co-analyzed calibration curve must be re-analyzed with a new curve including standards having higher concentrations of tebuconazole that bracket that of the sample.

7.1.3 Water

Quantitation of tebuconazole residue in water extracts is also performed by the calibration technique. Construct a new calibration curve of 0.5-, 1-, 2-, and 5-mg L^{-1} equivalent tebuconazole standard solutions for each set of analyses. Inject 5-μL aliquots of the standard solutions. The injection volume should be kept constant as the peak area varies with the injection volume by nitrogen–phosphorus detection. Plot the peak area against the injected concentrations of tebuconazole.

7.2 Recoveries, limits of detection, and limits of quantification

7.2.1 Plant material

Net recoveries of tebuconazole from matrices fortified at 0.01–7.0 mg kg^{-1} ranged from 78 to 116%. The limit of detection (LOD) based on control interferences in matrices ranged from 0.001 to 0.01 mg kg^{-1}. The limit of quantification (LOQ) based on recoveries was established at 0.01 mg kg^{-1}.

7.2.2 Soil

Recoveries from soil averaged 96% at 0.1 mg kg^{-1} and 99% at 0.01 mg kg^{-1}. The instrumental response was linear over the range 0.01–1 mg kg^{-1} equivalents in both

solvent and matrix. The LOD and LOQ were established at 0.01 mg kg^{-1}, but lower levels can be obtained by adjusting the final extract volume.

7.2.3 Water

With fortification levels between 0.5 and 5 μg L^{-1}, recoveries from blank water ranged from 92 to 113% with the LOQ being 0.5 μg L^{-1}.

7.3 Calculation of residues

7.3.1 Plant material

The amount of tebuconazole residue (R, mg kg^{-1}) in the sample is calculated by the following equation:

$$R = (\text{response ratio sample})/(\text{av. standard response ratio}) \times \text{std conc.} \times \text{dil. factor}$$

where

std conc. = mg kg^{-1} tebuconazole equivalent in final sample extract (i.e. 0.004 mg L^{-1} = 0.05 mg kg^{-1})
dil. factor = factor of dilution applied to final sample extract prior to IS addition

DropBooks

7.3.2 Soil

The amount of tebuconazole residue (R) is calculated by applying the response factor (RF) to a standard (std) calibration curve. Calculations are performed using the instrument software using the following equation:

$$R\ (\text{mg kg}^{-1}) = [(\text{area analyte/area IS} \times \text{conc. IS})/(\text{RF} \times \text{DF})]$$

where

IS = internal standard
RF = [(area std/area IS) × (conc. IS/conc. Std)]
DF = dilution factor (if applicable) = (initial volume/final volume)

7.3.3 Water

The amount of tebuconazole residue is calculated by using a least-squares fitting algorithm to generate the 'best' line which can be used to calculate the corresponding concentration for a given peak area or peak height. Calculate the slope and the intercept of the standard calibration curve.

Determine the concentration (C, mg L^{-1}) corresponding to each sample peak response using the intercept and slope from the above calculation:

$$C\ (\text{mg L}^{-1}) = [(\text{slope of regression curve}) \times (\text{sample peak area})] + (\text{intercept of curve})$$

where

C = concentration of tebuconazole in the final solution ($\mu g\ mL^{-1}$)

Calculate the concentration of tebuconazole in the water sample:

$$\text{Tebuconazole } (\mu g\ L^{-1}) = [C\ (mg\ L^{-1})] \times (V/G) \times 1000$$

where

V = final sample volume (mL)
G = original sample extraction volume (mL)

Reference

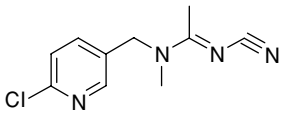
1. G.C. Mattern, C.I. Nuessle, D.L. Green, W.M. Leimkuehler, J.D. Philpot, R.J. Ness, and K.S. Billesbach, 'Accelerated field residue analysis of tebuconazole using Soxtec extraction and HPLC/electrospray tandem mass spectrometry (HPLC/ESI-MS-MS),' Presented at the Midwest Regional Meeting of the American Chemical Society, Osage Beach, MO, October 29, 1997.

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Acetamiprid

Materials to be analyzed	Cabbage, potato, radish (leaf), radish (root), grape, citrus, apple, pear, strawberry, cucurbits, green pepper, egg-plant, tomato, green tea (powder), green tea (leachate) and soil
Instrumentation	Gas-chromatographic determination for plant materials High-performance liquid chromatographic determination for soil

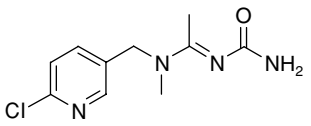
1 Introduction

Chemical name (IUPAC)	(*E*)-N^1-[(6-Chloro-3-pyridyl)methyl-N^2-cyano-N^1-methyl-acetamidine
Structural formula	
Empirical formula	$C_{10}H_{11}Cl\,N_4$
Molar mass	222.7
Melting point	98.9 °C
Vapor pressure	$<1\times10^{-6}$ Pa at 25 °C
Solubility	Water 4.25 g L^{-1} at 25 °C Readily soluble in organic solvents such as acetone, acetonitrile, chloroform, dichloromethane and methanol
Stability	Stable in an acidic to neutral aqueous solution. Unstable in strongly basic conditions Stable in most organic solvents
Use pattern	Acetamiprid is a neonicotinoid insecticide with outstanding systemic activities and a broad insecticidal spectrum. Acetamiprid controls diverse soil and foliar insect pests infesting cotton, sugar beet, vegetables, fruits and other major food crops by both contact and stomach action
Regulatory position	The residue definition for plant samples is acetamiprid only. In soil, it includes acetamiprid and three of its

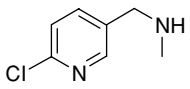
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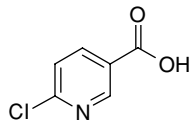
metabolites, (*E*)-N^2-carbamoyl-N^1-[(6-chloro-3-pyridyl)methyl-N^1-methylacetamidine (IM-1-2), *N*-methyl-[(6-chloro-3-pyridyl)methylamine (IM-1-4) and 6-chloronicotinic acid (IC-0)



IM-1-2



IM-1-4



IC-0

2 Outline of method

2.1 Plant

Plant materials are homogenized with methanol. Acetamiprid residue is extracted with dichloromethane by liquid–liquid partitioning. Dichloromethane is removed by rotary evaporation, and the residue is subjected to a clean-up procedure using Florisil PR column chromatography. The concentrated eluate is analyzed by gas chromatography (GC).

2.2 Soil

Soil sample is extracted with a mixture of methanol and 0.1 M ammonium chloride. Acetamiprid, IM-1-2 and IM-1-4 residues are extracted with dichloromethane under alkaline conditions. After adding diethylene glycol, dichloromethane in the extract is removed by rotary evaporation, and the residue is subjected to a cleanup procedure using Florisil PR column chromatography and then with a packed Extrelut 20 column.

IC-0 residue is cleaned up with a mixture of dichloromethane and acetone by liquid–liquid partitioning under neutral conditions and then extracted into diethyl ether under acidic conditions. The diethyl ether in the extract is removed by rotary evaporation and the residue is dissolved in buffer solution, which is subjected to a cleanup procedure using a Sep-Pak C_{18} Env. column.

The concentrated eluate is subjected to high-performance liquid chromatography (HPLC) analysis.

3 Apparatus

High-speed blender fitted with leak-proof glass jar and explosion-proof motor
Balances
Macerator (Polytron)
Laboratory mechanical shaker

Glass tube for column chromatography, 15-mm i.d., 30-mm length
Separatory funnels, 200-mL, 500-mL and 1-L
Filter paper
Erlenmeyer flask, 500-mL
Round-bottom flasks, 300-mL
Rotary vacuum evaporator
Stainless-steel centrifuge tube, 250-mL
Ultracentrifuge
pH meter
Packed column (Extrelut 20)
Packed column (Sep-Pak C_{18})
Packed column (Sep-Pak C_{18} Env.)
Gas chromatograph equipped with an electron capture detector
High-performance liquid chromatograph

4 Reagents

Methanol, guaranteed reagent grade
Celite (No. 545)
Sodium chloride, guaranteed reagent grade
n-Hexane, guaranteed reagent grade
Dichloromethane, guaranteed reagent grade
Sodium hydroxide, guaranteed reagent grade
Sodium sulfate, anhydrous, guaranteed reagent grade
Florisil, nonactivated (Florisil PR)
Ammonium chloride, guaranteed reagent grade
Diethylene glycol, guaranteed reagent grade
Hydrochloric acid, guaranteed reagent grade
Acetone, guaranteed reagent grade
Diethyl ether, guaranteed reagent grade
Acetonitrile, guaranteed reagent grade
Disodium hydrogenphosphate, guaranteed reagent grade
Citric acid, anhydrous
Nitrogen, repurified

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5 Sampling and preparation

5.1 Green tea

Grind leaves with dry-ice using a high-speed blender.

5.2 Fruits and vegetables

Cut into pieces with a kitchen knife.

6 Procedure

6.1 Extraction

6.1.1 Plant material

Homogenize 20 g of a prepared sample with 100 mL of methanol in a macerator for 3 min and shake for 30 min with a mechanical shaker. In the case of green tea (powder), soak 4 g of a prepared sample with 16 mL of distilled water for 2 h. Add 100-mL of methanol and shake for 30 min.

Filter the homogenate through a Celite layer (1–2 cm thickness) under reduced pressure. Wash the cake and vessel twice with 25 mL of methanol and filter the washings through the same Celite layer. Combine these filtrates and transfer to a 500-mL separatory funnel. Add 150 mL of 5% sodium chloride solution to the filtrate and wash twice with 100 mL of hexane for 10 min. Discard the hexane extract. Transfer the aqueous methanol to another 500-mL separatory funnel. In the case of green tea (leachate), soak 4 g of the ground sample in 240 mL of boiling water for 5 min. Filter the mixture through a filter paper and cool to ambient temperature. Remove half of the filtrate for further analysis (corresponds to 2 g of green tea dried). Add 5 g of sodium chloride and 120 mL of methanol to the filtrate and then wash twice with 100 mL of hexane for 10 min. Discard the hexane extract. Transfer the aqueous methanol into a 500-mL separatory funnel. Extract the solution with two portions of 100 mL of dichloromethane for 10 min. Collect the dichloromethane in a flask. In the case of citrus, wash the dichloromethane with 100 mL of 0.05 M sodium hydroxide solution for 5 min and discard the alkaline solution.

Pass the dichloromethane through a filter paper with anhydrous sodium sulfate and collect the dichloromethane in a 300-mL round-bottom flask. Add 1-g of Florisil PR and then evaporate dichloromethane to near dryness on a water-bath at ca 40 °C by rotary evaporation.

6.1.2 Soil

Recovery of acetamiprid, IM-1-2 and IM-1-4. Combine 20 g of the air-dried soil with 100 mL of a mixed solvent of methanol and 0.1 M ammonium chloride (4 : 1, v/v) in a 250-mL stainless-steel centrifuge tube, shake the mixture with a mechanical shaker for 30 min and centrifuge at 8000 r.p.m. for 2 min. Filter the supernatant through a Celite layer (1-cm thick) under reduced pressure into a 500-mL flask. Add a second 100 mL of mixed solvent to the residue and then extract and filter in the same manner. Combine the filtrates and add 150 mL of distilled water with 1 g of sodium chloride. Transfer the aqueous methanol solution into a 1-L separatory funnel and shake the solution with 200 mL of dichloromethane for 5 min. Collect the dichloromethane in a flask and adjust the pH of aqueous methanol to 13 with sodium hydroxide. Extract the solution with two portions of 200 mL of dichloromethane for 5 min. Combine the dichloromethane extracts and pass through a filter paper with anhydrous sodium sulfate. Add 0.5 mL of diethylene glycol and then concentrate the dichloromethane extract to about 0.5 mL on a water-bath at ca 40 °C by rotary evaporation.

Recovery of IC-0. Combine 20 g of the air-dried soil with 100 mL of a mixed solvent of methanol and 0.1 M ammonium chloride (4 : 1, v/v) in a 250-mL stainless-steel centrifuge tube, shake the mixture with a mechanical shaker for 30 min and centrifuge at 8000 r.p.m. for 2 min. Filter the supernatant through a Celite layer (1-cm thick) under reduced pressure into a 500-mL flask. Add 100 mL of mixed solvent of methanol and 0.5 M sodium hydroxide solution (4 : 1, v/v) to the residue and then extract and filter in the same manner. Combine the filtrates and concentrate to approximately 40 mL on a water-bath at ca 40 °C by rotary evaporation. Add 10 mL of distilled water and adjust the pH of the aqueous layer to 7 with hydrochloric acid. Transfer the aqueous solution into a 200-mL separatory funnel and shake the solution with 50 mL of mixed solvent of dichloromethane and acetone (1 : 1, v/v) for 5 min. Discard the mixed solvent and adjust the pH of the aqueous layer to 1.5 with hydrochloric acid. Extract the solution with three portions of 50 mL of diethyl ether. Combine the diethyl ether extracts and dry over anhydrous sodium sulfate. Concentrate to dryness on a water-bath at ca 40 °C by rotary evaporation.

6.2 Cleanup

6.2.1 Plant materials

Transfer the residue with 1 g of Florisil PR on to the top of the column packed with 9 g of Florisil PR with the aid of hexane. Rinse the column with 150 mL of hexane-acetone (4 : 1, v/v). Elute acetamiprid with 120 mL of a mixed solvent of acetone-hexane (1 : 1, v/v) and concentrate the eluate to near dryness by rotary evaporation at 40 °C. Dissolve the residue with 5 mL of distilled water and apply the solution to the top of the packed Sep-Pak C_{18} column pretreated with 20 mL each of methanol and distilled water. Elute acetamiprid with 30 mL of a mixed solution of water-acetonitrile (17 : 3, v/v). Concentrate to dryness on a water-bath at ca 40 °C by rotary evaporation. Prepare the GC-ready sample by dissolving the residue in acetone.

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6.2.2 Soil

Cleanup procedures for acetamiprid, IM-1-2 and IM-1-4. Dilute the concentrate with 10 mL of distilled water and apply the solution to an Extrelut 20 column, equilibrate for 20 min at ambient temperature and pass 100 mL of dichloromethane through the column. Collect the eluate and add 0.5 mL of diethylene glycol and then concentrate the dichloromethane to about 0.5 mL by rotary evaporation. Prepare the HPLC-ready sample solution by dissolving the residue in 50% aqueous acetonitrile.

Cleanup procedure for IC-0. Dissolve the residue with 10 mL of pH 5 phosphate buffer solution and apply the solution to the top the Sep-Pak C_{18} Env. column pretreated with 10 mL each of methanol and distilled water. Discard the passed solution and elute IC-0 with 15 mL of a second buffer solution. Add 35 mL of distilled water and adjust the pH of solution to 1.5 with hydrochloric acid. Extract the solution with three portions of 50 mL of diethyl ether. Combine the diethyl ether extracts and dry over anhydrous sodium sulfate. Concentrate to dryness on a water-bath at ca 40 °C

by rotary evaporation. Prepare the HPLC-ready sample solution by dissolving the residue in 50% aqueous acetonitrile solution.

6.3 Determination

6.3.1 Plant materials

Inject an aliquot of the GC-ready sample solution into the gas chromatograph.

Operating conditions

Gas chromatograph	Model GC-14B, Shimadzu
Column	5% PEG HT/Chromosorb W HP column, 60–80 mesh, 3.2-mm i.d., 1.0-m length short column
Column temperature	260 °C
Injection port temperature	320 °C
Detector	Electron capture detector
Detector temperature	320 °C
Gas flow rates	Nitrogen carrier gas, column head pressure 1.5 kg cm^{-2}
Injection volume	2 μL

6.3.2 Soil

Inject an aliquot of the HPLC-ready sample solution into the high-performance liquid chromatograph.

Operating conditions

High-performance liquid chromatograph	Model LC-10AD, Shimadzu
Column	Stainless-steel column, 4.6-mm i.d., 150 mm-length
Stationary phase	Inertsil ODS-3

(1) Determination of acetamiprid, IM-1-2 and IM-1-4

Mobile phase	(A) 0.1 M ammonium acetate adjusted to pH 8.5 with aqueous ammonia (B) Acetonitrile
Gradient rate	0–20 min 85% A–15% B to 70% A–30% B 20–22 min 70% A–30% B to 30% A–70% B 22–30 min 85% A–15% B
Flow rate	1.0 mL min^{-1}
Column temperature	50 °C
Detection	UV detector (SPD-10AV) at 270 nm (IM-1-4) for initial 11 min and then change at 246 nm (acetamiprid, IM-1-2) for 19 min
Injection volume	25 μL

(2) Determination of IC-0

Mobile phase	Acetonitrile–1% acetic acid (1 : 4, v/v)
Flow rate	1.0 mL min^{-1}
Column temperature	50 °C
Detection	UV detector (SPD-10AV) at 270 nm (IC-0)
Injection volume	25 μL

7 Evaluation

7.1 Method

7.1.1 Plant materials

Quantification is performed by the calibration technique. Construct a new calibration curve with acetamiprid standard solutions using acetone for each set of analyses. Inject 2-μL aliquots of the standard solution containing acetamiprid from 0.04 to 1 ng in 2μL of acetone. The acetamiprid peak usually appears at a retention time around 4 min. Plot the peak height against the injected amount of acetamiprid.

7.1.2 Soil

Quantification is performed by the calibration technique. Construct a new calibration curve with the mixed standard solutions of acetamiprid, IM-1-2 and IM-1-4 for each set of analyses. Inject 25-μL aliquots of the standard solutions containing compounds from 1 to 10 ng in 25 μL of 50% aqueous acetonitrile. With regard to IC-0, prepare the calibration curve in the same manner. The retention times are around 8 min for IM-1-4 and IC-0, 12 min for IM-1-2 and 20 min for acetamiprid. Plot the peak area against the injected amount of each standard.

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7.2 Recoveries, limit of detection and limit of determination

7.2.1 Plant materials

With a fortification level of 0.1 mg kg^{-1}, recoveries from untreated plant matrices ranged from 90 to 104%. The limit of detection (LOD) was 0.005 mg kg^{-1} (fruits and vegetables). With regard to green tea (powder and leachate), the method recoveries were 95 and 98%, respectively, at the 0.5 mg kg^{-1} fortification level. The LOD was 0.05 mg kg^{-1}.

7.2.2 Soil

At the fortification levels at 0.1 and 0.2 mg kg^{-1}, recoveries of acetamiprid, IM-1-2, IM-1-4 and IC-0 from soils ranged from 70 and 95%. The LOD for each compound was 0.01 mg kg^{-1}.

7.3 Calculation of residues

The amount of acetamiprid and its related compounds (R, mg kg^{-1}) in the sample is calculated by the following equation:

$$R = C \times V / G$$

where

C = concentration of compound in the final solution (μg mL^{-1})
V = final sample volume (mL)
G = original sample weight (g)

8 Important points

Since IM-1-4 is a volatile compound, diethylene glycol should be added to the solution containing IM-1-4 to reduce the amount of sample lost in the concentration step under reduced pressure.

Further reading

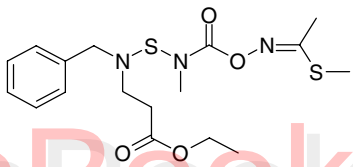
M. Tokieda, K. Iiyoshi, K. Sugioka, and T. Gomyo, *J. Pestic. Sci.,* **22**, 129 (1997).
M. Tokieda, T. Tanaka, M. Ozawa, and T. Gomyo, *J. Pestic. Sci.,* **23**, 296 (1998).

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Alanycarb

Materials to be analyzed	Apple, cereals, citrus, corn, cotton, grapevine, peach, peanut, pear, potato, sorghum, soybean, sugar beet, tea, tobacco, cabbage, onion, turf grass, woody plant, water, soil and air
Instrumentation	Gas-chromatographic determination for plant materials High-performance liquid chromatographic determination for water, soil and air

1 Introduction

Chemical name (IUPAC)	Ethyl (*Z*)-*N*-benzyl-*N*-{[methyl(1-methylthioethylidene-aminooxycarbonyl)amino]thio}-β-alaninate
Structural formula	
Empirical formula	$C_{17}H_{25}N_3O_4S_2$
Molar mass	399.5
Melting point	46.8–47.2 °C
Vapor pressure	$<4.7 \times 10^{-6}$ Pa at 20 °C
Solubility	Water 20 mg L^{-1} at 20 °C Readily soluble in polar organic solvents
Stability	Stable in neutral and weakly basic conditions Unstable in strongly acidic or basic conditions Stable in most of organic solvents such as acetone, acetonitrile and carbon tetrachloride
Other properties	Undergoes hydrolysis to yield methomyl oxime in alkaline solutions
Use pattern	Alanycarb is an oxime carbamate insecticide with outstanding control of many important *Lepidoptera* pests of agricultural crops. Major features of this product include mammalian safety, excellent residual activity and absence of phytotoxicity on many agricultural crops

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Regulatory position	The residue definition includes alanycarb and its metabolite, methomyl (determined as methomyl oxime)

2 Outline of method

Plant and soil materials are homogenized with a mixture of borate buffer and acetone. Alanycarb residue is extracted into acetone and collected in dichloromethane by liquid–liquid partitioning with water. Alanycarb residue is directly extracted into dichloromethane. The dichloromethane in the extract is removed by rotary evaporation, and the residue is dissolved in carbon tetrachloride, which is subjected to a cleanup procedure using silica gel column chromatography. In the case of soil samples, the concentrated eluate is subjected to high-performance liquid chromatography (HPLC) analysis. The residue in plant materials is hydrolyzed by heating in 0.1 N aqueous sodium hydroxide to yield methomyl oxime, which can be recovered by ethyl acetate solvent extraction. The ethyl acetate is removed by rotary evaporation and the residue is dissolved in acetone for gas chromatography (GC) analysis. The water sample is passed through an Empore extraction disk from which alanycarb is eluted with acetonitrile. The extract is dried by rotary evaporation and the residue is dissolved in acetonitrile for HPLC analysis.

In the case of residue analysis in air, a known amount of air is passed through a sampling cartridge. The adsorbed alanycarb is extracted with acetonitrile. The acetonitrile in the extract is removed by rotary evaporation, the residue is dissolved in acetonitrile and the solution is subjected to HPLC analysis.

3 Apparatus

High-speed blender fitted with leak-proof glass jar and explosion-proof motor
Laboratory mechanical shaker
Filter paper, 9-cm diameter
Ultrasonic cleaner
Empore extraction disk, C_{18}, 47-mm
Erlenmeyer flask, 300-mL
Filtration flask, 1-L
Round-bottom flasks, 100-, 300- and 500-mL
ORBO-42 tube: A sampling cartridge filled with two portions (100-mg front bed and 50-mg backup bed) of adsorbent (porous styrene–divinylbenzene copolymer), 8-mm i.d., 100-mm length
Rotary vacuum evaporator, 35 °C bath temperature
Separatory funnel, 500-mL
Gas chromatograph equipped with a flame photometric detector
High-performance liquid chromatograph

4 Reagents

Methyl thioacetohydroxamate standard, Wako Pure Chemical Industries, Ltd
Acetone, reagent grade
Acetonitrile, reagent grade
Methanol, reagent grade
Dichloromethane, reagent grade
Ethyl acetate, reagent grade
n-Hexane, reagent grade
Carbon tetrachloride, reagent grade
Potassium chloride solution, aqueous solution, 135-g L^{-1}
Sodium sulfate, anhydrous
Ethylene glycol, reagent grade
Sodium dodecyl sulfate, reagent grade
Sodium hydroxide, reagent grade
Boric acid, reagent grade
pH 10.0 borate buffer: A mixture of 50 mL of 0.2 N boric acid–potassium chloride (H_3BO_3 12.405 g, KCl 14.912 g in 1 L of distilled water) and 43.9 mL of 0.2 M sodium hydroxide, diluted with distilled water to 100 mL
Sulfuric acid, reagent grade
Silica gel, Wako gel C-200, 100–200 mesh
Celite, No. 545
Air, synthetic
Nitrogen, repurified
Hydrogen, repurified
pH test paper, universal (pH 1–11)

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5 Sampling and preparation

No specific sample preparation and processing are required for this method.

6 Procedure

6.1 Extraction

6.1.1 Plant materials

Homogenize 50 g of a prepared sample with a solution containing 50 mL of borate buffer (pH 10) and 50 mL of acetone in a blender for 5 min. Pour the homogenate into an Erlenmeyer flask, add 50 mL of acetone and shake the flask for 10 min using a shaker. Filter the aqueous acetone extract through a 25G-4 glass filter overlaid with 3 g of Celite. Wash the residue on the filter with 50 mL of acetone. Combine the filtrates and remove acetone by rotary evaporation. Transfer the residue with 5 mL of 4% sodium dodecyl sulfate aqueous solution into a separatory funnel, extract the solution with two portions of 50 mL of dichloromethane and collect the organic

extracts in a flask. Filter the combined solvent extracts, together with the washings of the collection flask, through anhydrous sodium sulfate into a 300-mL flask. Remove dichloromethane by rotary evaporation. Dissolve the residue in 10 mL of carbon tetrachloride.

6.1.2 Soil

Combine 50 g of the air-dried soil with 100 mL of acetone and shake the mixture with a mechanical shaker for 15 min. Filter the mixture through a fluted filter paper into a 500-mL flask. Wash the residue on the filter with 50 mL of acetone. Combine the filtrates and remove acetone by rotary evaporation. Transfer the residue with 150 mL of a potassium chloride solution into a separatory funnel, extract the solution with two portions of 50 mL of dichloromethane and collect the organic extracts in a flask. Filter the combined solvent extracts, together with the washings of the collecting flask, through anhydrous sodium sulfate into a 300-mL flask. Remove dichloromethane by rotary evaporation. Dissolve the residue in 10 mL of carbon tetrachloride.

6.1.3 Water

Filter 1 L of water sample through a filter paper. Place an Empore extraction disk in a Millipore extraction funnel. Rinse the disk with 10 mL of ethyl acetate, dichloromethane, and acetonitrile, successively. Dry the disk under vacuum and then rinse the disk with 10 mL of methanol and 20 mL of deionized water by vacuum filtration. Pass the prefiltered sample through the disk and elute alanycarb with two portions of 10 mL of acetonitrile. Transfer the eluates through anhydrous sodium sulfate into a 50-mL flask. Remove acetonitrile by rotary evaporation. Dissolve the residue in 1 mL of acetonitrile.

6.1.4 Air

Just before sampling, remove the seals covering both ends of the ORBO tube using an ORBO tube cutter. Place the sampling cartridge between aspirator and flow meter and connect by silicon tubes. Adjust the suction rate to 1000 mL min^{-1}. This rate should be kept constant throughout the sampling period. Pass 40 L of air through the system, and terminate sampling by removing the tube between the cartridge and aspirator. Transfer each adsorbent (a 100-mg front bed and a 50-mg backup bed) into a separate vial and add 20 mL of acetonitrile. Extract the trapped alanycarb from the adsorbent into acetonitrile by ultrasonic extraction for 10 min. Take a 10-mL aliquot of the acetonitrile extract and concentrate to near dryness by rotary evaporation. Dissolve the residue in 1 mL of acetonitrile.

6.2 Cleanup

For plant and soil samples, transfer the carbon tetrachloride solution into a glass column packed with 7 g of silica gel saturated in carbon tetrachloride. Rinse

the column, first with 2 mL of carbon tetrachloride and then with 20 mL of hexane–ethyl acetate (3 : 2, v/v). Elute alanycarb with 70 mL of the same hexane–ethyl acetate solution. In the case of a soil sample, concentrate the eluate to dryness by rotary evaporation and prepare the HPLC-ready sample solution by dissolving the residue in 5 mL of acetonitrile. For a plant sample, dissolve the residue in 1 mL of acetone.

6.3 Saponification

In the case of plant samples, the aforementioned acetone solution is heated with 30 mL of 0.1 N aqueous sodium hydroxide at 80 °C for 40 min in a water-bath to hydrolyze alanycarb to methomyl oxime. Cool the hydrolysate to room temperature, acidify to pH 2–3 by adding 1 N sulfuric acid, extract the solution in a separatory funnel with two portions of 50 mL of ethyl acetate and collect the organic extracts in a flask. Filter the combined solvent extracts, together with the washings of the collecting flask, through anhydrous sodium sulfate into a 300-mL flask. Add 0.2 mL of 2% ethylene glycol in acetone, remove ethyl acetate by rotary evaporation, and prepare the GC-ready sample solution by dissolving the residue in 1 mL of acetone.

6.4 Determination

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6.4.1 Plant materials

Inject an aliquot of the GC-ready sample solution into the gas chromatograph.

Operating conditions

Gas chromatograph	Model 7-AG, Shimadzu
Column	Fused-silica capillary column, 0.53-mm i.d., 30-m length; coated with DB-WAX
Column temperature	170 °C
Injection port temperature	250 °C
Detector	Flame photometric detector, FPD-7
Detector temperature	250 °C
Gas flow rates	Nitrogen carrier gas, 60 mL min^{-1} Hydrogen, 60 mL min^{-1} Air, 60 mL min^{-1}
Injection volume	10 μL

6.4.2 Soil, water and air

Inject an aliquot of the HPLC-ready sample solution into the high-performance liquid chromatograph.

Operating conditions

High-performance liquid chromatograph	Model LC-6A, Shimadzu
Column	Stainless-steel column, 4-mm i.d., 250-mm length
Stationary phase	Zorbax BP ODS
Mobile phase	Acetonitrile–water (3 : 2, v/v)
Flow rate	1.0 mL min^{-1}
Column temperature	50 °C
Detection	UV detector at 240 nm
Injection volume	25 μL

7 Evaluation

7.1 Method

7.1.1 Plant material

Quantitation is performed by the calibration technique. Construct a new calibration curve with methomyl oxime standard solutions (0.2, 0.4, 0.6, 0.8 and 1.0 μg mL^{-1} in acetone) for each set of analyses. Plot the peak area against the injected amount of methomyl oxime on logarithmic paper. As the amount of alanycarb is measured in terms of its oxime derivative, a conversion factor of 3.8 (the molecular weight ratio of alanycarb to methomyl oxime) should be applied to obtain the net amount. The injection volume should be kept constant as the peak area varies with the injection volume in flame photometric detection. Before each set of measurements, check the GC system by injecting more than one standard solution containing ca 2–10 ng of methomyl oxime.

Recommendation: Inject standard solutions (0.2, 0.4, 0.6, 0.8 and 1.0 μg mL^{-1} in acetone) and sample solutions alternately rather than constructing the calibration curve in advance.

7.1.2 Soil, water and air

Quantitation is performed by the calibration technique. Construct a new calibration curve with alanycarb standard solutions for each set of analyses. Inject 25-μL aliquots of the standard solutions (2.0, 4.0, 6.0, 8.0 and 10.0 μg mL^{-1} in acetonitrile). The retention time is around 5.1 min. Plot the peak area against the injected amount of alanycarb.

7.2 Recoveries, limit of detection and limit of determination

7.2.1 Plant material

With fortification levels between 0.05 and 0.5 mg kg^{-1}, recoveries from untreated plant matrices ranged from 89 to 92% with the limit of determination being 0.01 mg kg^{-1}.

7.2.2 Soil

With a fortification level of 0.1 mg kg^{-1}, recoveries from blank soils ranged from 75 to 89% with the limit of determination being 0.05 mg kg^{-1}.

7.2.3 Water

With a fortification level of 0.01 mg kg^{-1}, recoveries from blank water ranged from 85 to 105% with the limit of determination being 0.0005 mg kg^{-1}.

7.2.4 Air

With fortification levels between 400 and 1000 ng in 100 mg of adsorbent, recoveries from adsorbent ranged from 94 to 102% with the limit of determination being 0.010 mg L^{-1}.

7.3 Calculation of residues

7.3.1 Plant material

The amount of alanycarb residue (R, mg kg^{-1}) in the sample is calculated with the following equation:

$$R = C \times V/G \times 3.8$$

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where

C = concentration of methomyl oxime in the final solution (μg mL^{-1})
V = final sample volume (mL)
G = original sample weight (g)
3.8 = ratio of molecular weight of alanycarb to that of methomyl oxime

7.3.2 Soil, water and air

The amount of alanycarb residue (R, mg kg^{-1} or mg L^{-1}) in the sample is calculated with the following equation:

$$R = C \times V/G$$

where

C = concentration of alanycarb in the final solution (μg mL^{-1})
V = final sample volume (mL)
G = original sample weight (g)

8 Important points

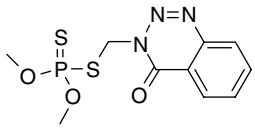
In the case of crop residues, GC determination is carried out on the hydrolyzed product, i.e., methomyl oxime, instead of alanycarb to make effective use of its substantially higher response to the flame photometric detector. In order to prevent vaporization loss of methomyl oxime, ethylene glycol must be added prior to concentration in Section 6.3. In all other concentration operations, full account must also be taken of the high volatility of both alanycarb and methomyl oxime, especially in the process of removal of the last traces of solvents. Alanycarb residue in the sample is stable under storage condition at −20 °C for at least 100 days.

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Azinphos-methyl

Materials to be analyzed	Apples and pears
Instrumentation	High-performance liquid chromatography/triple stage quadrupole mass spectrometry (LC/MS/MS)

1 Introduction

Chemical name (IUPAC)	(*S*)-(3,4-Dihydro-4-oxobenzo[*d*]-[1,2,3]-triazin-3-ylmethyl) *O*,*O*-dimethyl phosphorodithioate
Structural formula	
Empirical formula	$C_{10}H_{12}N_3O_3S_2$
Molar mass	317.3
Melting point	60–63 °C
Vapor pressure	1.6×10^{-6} mmHg at 20 °C
Solubility	Water 28 mg L^{-1} at 20 °C Readily soluble in polar organic solvents
Stability	Half-life at 22 °C for pH 4, 7 and 9 is 87, 50 and 4.1 days, respectively
Other properties	Azinphos-methyl is unstable in basic conditions and can degrade to benzazimide, hydroxymethyl-benzazimide, mercaptomethylbenzazimide or bis(benzazimide-*N*-methyl) sulfide
Use pattern	Azinphos-methyl is an organophosphorus insecticide used to control chewing and sucking mites and insects such as aphids and scale. Azinphos-methyl has both contact and stomach action. Azinphos-methyl is a cholinesterase inhibitor and interferes with the nervous system
Regulatory position	The residue definition for crops includes only azinphos-methyl

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2 Outline of method

Plant material is homogenized in acetone followed by addition of water. The filtered extract is diluted with acetone–water (2 : 1 v/v) and filtered through a syringe filter. The sample extract is diluted 1 : 1 with a deuterated azinphos-methyl internal standard and analyzed using LC/MS/MS in the positive-ion selected reaction monitoring (+SRM) mode.

3 Apparatus

Buchner funnels, 9-cm
Degasser for high-performance liquid chromatography (HPLC) mobile phase
Filters, GF/A, 9-cm diameter
Filter cartridges, GD/X, 25-mm, 0.45-μm
Graduated mixing cylinder, 250-mL
Heater for HPLC column
HPLC, ConstaMetric 3500 MS and ConstaMetric 3200 MS
Mass spectrometer, TSQ 7000 with atmospheric pressure ionization (API) electrospray interface
Robotcoupe, Model RSI 25
Syringes, Luer lock, 10-mL
Tekmar Tissumizer Model SDT 1810 S1 with SDT-S25N probe

4 Reagents

Acetonitrile, pesticide grade or HPLC grade
Acetone, pesticide grade or HPLC grade
Methanol, pesticide grade or HPLC grade
Argon, purity ≥99%
Nitrogen, purity ≥99%

5 Sampling and preparation

Add approximately 1 kg of the frozen raw agricultural commodity (RAC) to a Robotcoupe or equivalent sample processor. Add about an equal portion of pelletized dry-ice to the sample in the processor. Macerate the combined dry-ice and RAC until a homogeneous mixture is obtained.

6 Procedure

6.1 Extraction of plant material

Weigh 20 g of homogenized sample into a 300-mL tall-form beaker. Begin recovery samples at this point by fortifying the control tissue matrix samples. Add 120 mL of acetone, and homogenize the mixture for approximately 5 min followed by the addition of 60 mL of laboratory-grade water with another 2 min of homogenization.

Vacuum filter the contents of the beaker through a 9-cm GF/A filter contained in a 9-cm Buchner funnel and collect the filtrate in a 250-mL graduated mixing cylinder. Wash the extraction beaker with 60 mL of acetone–water (2 : 1, v/v), and use this to rinse the filter cake, adding the washings to the graduated cylinder. Dilute the sample extract to 250 mL with acetone–water (2 : 1 v/v). Remove the plunger from a 10-mL disposable Luer lock syringe, and attach a 25-mm, 0.45-μm Teflon GD/X filter cartridge to the end of the syringe barrel. Transfer approximately 4 mL of the 250 mL of solution into the syringe/filter apparatus. Replace the plunger in the syringe barrel, force the solution through the filter and collect about 4 mL in a clean glass vial with a Teflon lined lid for storage prior to analysis. The resulting solution in the glass vial represents 0.080 μg mL^{-1} azinphos-methyl per g matrix per mL dissolved in acetone–water (2 : 1 v/v).

6.2 Determination

6.2.1 Sample/internal standard solution

Combine a 0.5-mL aliquot of the final sample extract or a 0.020 μg mL^{-1} azinphos-methyl standard solution in acetone–water (2 : 1 v/v) with 0.5 mL of a 0.040 μg mL^{-1} deuterated internal standard solution in methanol–water (2 : 3 v/v) in an HPLC autosampler vial. Combination may be made using other volumes as long as the solutions are combined 1 : 1 (v/v). Inject 200 μL from the 0.020 and 0.040 μg mL^{-1} standard/internal standard solution. Inject 200 μL from each of the 10 sample extract/internal standard solutions. Inject 200 μL from another 0.020 and 0.040 μg mL^{-1} standard/internal standard solution.

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6.2.2 Instrumental setup

Thermo Separations Products HPLC system with an autosampler, solvent degasser and column heater interfaced to a Finnigan MAT 7000 mass spectrometer or equivalent instrumentation optimized for the detection of azinphos-methyl.

HPLC conditions

Column	Phenomenex Columbus 5-μm C_{18} 50 × 4.6-mm i.d. at 30 °C
Solvent A	0.1% formic acid in water
Solvent B	Methanol
Injection volume	200 μL
Flow rate	1.5 mL min^{-1}
Split ratio	1 : 6 post-column (200–250 μL min^{-1} through interface)

Mobile phase program (all flow rates 1.5 mL min^{-1} and all gradients are linear):

Time (min)	% A	% B	Hold (min)
Initial	60	40	1.00
5.00	20	80	1.00
6.10	0	100	1.90
8.10	60	40	1.90

Total run time	10.0 min
Retention time of azinphos-methyl	4.2 min
Diversion window to interface	2.0–6.0 min

Mass spectrometer conditions

Mode	Positive-ion single reaction monitoring	
Interface	Finnigan MAT API I ESI (electrospray ionization)	
Spray voltage	4.5 kV	
Sheath gas	Nitrogen at 100 psi	
Auxiliary gas	Nitrogen at 30 psi	
Capillary temperature	235 °C	
Manifold temperature	70 °C	
Collision gas	Argon at 2.5 mTorr	
Total scan time	1 s	
Q1MS resolution voltage added	None	
Q3MS resolution voltage added	5.0 V	
Parent masses	Azinphos-methyl:	mass axis calibrated to *m/z* 318
	[$^2H_6$]Azinphos-methyl:	mass axis calibrated to *m/z* 324
Ions detected	Azinphos-methyl:	*m/z* 132 daughter of *m/z* 318
		m/z 160 daughter of *m/z* 318
		m/z 261 daughter of *m/z* 318
	[$^2H_6$]Azinphos-methyl:	*m/z* 132 daughter of *m/z* 324
		m/z 160 daughter of *m/z* 324
		m/z 267 daughter of *m/z* 324
Ions used for quantitation	Azinphos-methyl:	*m/z* 160 daughter of *m/z* 318
	[$^2H_6$]Azinphos-methyl:	*m/z* 160 daughter of *m/z* 324

7 Evaluation

7.1 Response factor

Compare the ratio (response factor) of the peak areas of azinphos-methyl to [$^2H_6$]azinphos-methyl of each sample extract to the average ratio of the respective peak areas of the standard solutions on either side of the set of 10 samples.

7.2 Recoveries, limit of detection and limit of quantification

With fortification levels between 0.01 and 0.1 mg kg^{-1}, recoveries from untreated plant matrices ranged from 70 to 113%. The limit of detection is 0.002 mg kg^{-1} and the limit of quantification is 0.01 mg kg^{-1}.

7.3 Calculation of residues

The amount of azinphos-methyl residue (R, mg kg^{-1}) in the sample is calculated by the following equation:

$$R = \frac{\text{response factor sample}}{\text{av. standard response factor}} \times \text{std conc.} \times \text{dilution factor}$$

8 Important points

The official enforcement method is a GC method, but the method described here is quicker and more robust. Approximately 36 samples can be analyzed within 24 h.

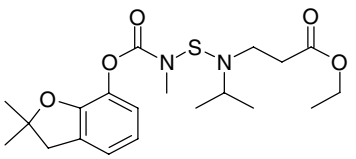
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Benfuracarb

Materials to be analyzed	Citrus, cotton, melon, watermelon, banana, tomato, eggplant, onion, cabbage, carrot, chicory, leek, maize, hazelnut, potato, rice (straw, grain), air, sweet corn, soybean, French bean, sugar beet, flowers and ornamentals, sunflower, tobacco, soil and water
Instrumentation	Gas-chromatographic determination for plant materials High-performance liquid chromatographic determination for water and soil

1 Introduction

Chemical name (IUPAC)	Ethyl *N*-[2,3-dihydro-2,2-dimethylbenzofuran-7-yloxycarbonyl(methyl)aminothio]-*N*-isopropyl-β-alaninate
Structural formula	
Empirical formula	$C_{20}H_{30}N_2O_5S$
Molar mass	410.5
Boiling point	110 °C/0.023 mmHg
Vapor pressure	$<1 \times 10^{-5}$ Pa at 20 °C
Solubility	Water 7.74 mg L^{-1} at 20 °C Readily soluble in polar organic solvents
Stability	Stable in a neutral to basic aqueous solution Unstable in strongly acidic or basic conditions Stable in most of organic solvents such as benzene, acetonitrile and carbon tetrachloride
Other properties	The N–S bond in the molecule is unstable in aqueous acidic solution; the N–S bond is also susceptible to thiolytic cleavage when the sulfur atom is attacked by sulfhydryl-containing agents
Use pattern	Benfuracarb is a carbamate insecticide with an outstanding systemic and broad insecticidal spectrum. Benfuracarb controls diverse soil and foliar insect pests

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	and also nematodes infesting rice, maize, sugar beet, vegetables, fruits and other major food crops. On these pests, benfuracarb works by both or either of contact and stomach action
Regulatory position	The residue definition includes benfuracarb and two of its metabolites, carbofuran and 3-hydroxycarbofuran

2 Outline of method

Plant and soil materials are homogenized with a mixture of acetonitrile–phosphate buffer and silver nitrate solution; for water samples, this step is omitted. The silver nitrate solution is used to prevent the N–S bond cleavage during homogenization and extraction. Benfuracarb residue is extracted into acetonitrile and collected in dichloromethane by liquid–liquid partitioning with water. Benfuracarb residue is directly extracted into dichloromethane. The dichloromethane in the extract is removed by rotary evaporation, the residue is dissolved in carbon tetrachloride and the solution is subjected to a cleanup procedure using Florisil and silica gel column chromatography. The concentrated eluate is subjected to gas chromatography (GC) analysis (for plant materials) and high-performance liquid chromatography (HPLC) analysis (for soil and water samples).

In residue analysis in air, a known amount of air is passed through a sampling cartridge. The trapped benfuracarb is extracted with acetonitrile. The solvent extract is removed by rotary evaporation, the residue is dissolved in acetonitrile and the solution is subjected to HPLC analysis.

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3 Apparatus

High-speed blender fitted with leak-proof glass jar and explosion-proof motor
Laboratory mechanical shaker
Filter paper, 9-cm diameter
Ultrasonic cleaner
Erlenmeyer flask, 300-mL
Filtration flask, 1-L
Round-bottom flasks, 100-, 300- and 500-mL
ORBO-42 tube: A sampling cartridge filled with two portions (100-mg front bed and 50-mg backup bed) of adsorbent (porous styrene–divinylbenzene copolymer), 8-mm i.d., 100-mm length
Ultrasonic cleaner
Rotary vacuum evaporator, 35 °C bath temperature
Separatory funnel, 500-mL
Gas chromatograph equipped with a flame thermionic detector
High-performance liquid chromatograph

4 Reagents

Acetonitrile, reagent grade
Dichloromethane, reagent grade
Ethyl acetate, reagent grade
n-Hexane, reagent grade
Silver nitrate solution, aqueous solution, 0.1 M
Carbon tetrachloride, reagent grade
Potassium chloride solution, aqueous solution, 135 g L^{-1}
Sodium sulfate, anhydrous
Florisil, 100–200 mesh
Silica gel, Wako gel C-200, 100–200 mesh
Air, synthetic
Nitrogen, repurified
Hydrogen, repurified

5 Sampling and preparation

No specific sample preparation and processing are needed for this method.

6 Procedure

6.1 Extraction

6.1.1 Plant material

Homogenize 50 g of a prepared sample with a solution containing 25 mL of phosphate buffer (pH 8.0), 2 mL of 0.1 M silver nitrate solution and 50 mL of acetonitrile in a blender for 5 min. In the case of rice grain, mill the unpolished rice grains with a coffee mill and sieve through a 42-mesh screen. Place 50 g of the milled sample in an Erlenmeyer flask and add 50 mL of acetonitrile and 2 mL of 0.1 M silver nitrate solution. Pour the homogenate or milled sample (rice grain) into an Erlenmeyer flask, add 100 mL of acetonitrile and shake the flask for 10 min using a shaker. Filter the aqueous acetonitrile extract through a 25G-4 glass filter. Wash the residue on the filter with 50 mL of acetonitrile. Combine the filtrates and remove acetonitrile by rotary evaporation. Transfer the residue with 150 mL of potassium chloride solution into a separatory funnel, extract the solution with two portions of 50 mL of dichloromethane and collect the organic phase in a flask. Filter the combined solvent extracts, together with the washings of the collection flask, through anhydrous sodium sulfate into a 300-mL flask. Remove the dichloromethane by rotary evaporation. Dissolve the residue in 20 mL of carbon tetrachloride.

6.1.2 Soil

Combine 50 g of the air dried soil with 150 mL of acetonitrile and 2 mL of 0.1 M silver nitrate solution and shake the mixture with a mechanical shaker for 30 min. Filter the

mixture through a fluted filter paper into a 500-mL flask. Wash the residue on the filter with 50 mL of acetonitrile. Combine the filtrates and remove acetonitrile by rotary evaporation. Transfer the residue with 150 mL of a potassium chloride solution into a separatory funnel, extract the solution with two portions of 50 mL of dichloromethane and collect the organic extracts in a flask. Filter the combined solvent extracts, together with the washings of the collecting flask, through anhydrous sodium sulfate into a 300-mL flask. Remove dichloromethane by rotary evaporation. Dissolve the residue in 20 mL of carbon tetrachloride.

6.1.3 Water

Transfer 250 mL of water into a 500-mL separatory funnel. Extract the sample with two portions of 50 mL of dichloromethane and collect the extracts in a flask. Filter the combined extracts, together with the washings of the collecting flask, through anhydrous sodium sulfate into a 300-mL flask. Remove the dichloromethane by rotary evaporation. Dissolve the residue in 20 mL of carbon tetrachloride.

6.1.4 Air

Just before sampling, remove the seals covering both ends of the ORBO tube using an ORBO tube cutter. Place the sampling cartridge between the aspirator and flow meter and connect by silicon tubes. Adjust the suction rate to 1000 mL min^{-1}. This rate should be kept constant throughout the sampling period. Pass 40 L of air through the system and terminate sampling by removing the tube between the cartridge and aspirator. Transfer each adsorbent (a 100-mg front bed and a 50-mg backup bed) into a separate vial and add 20 mL of acetonitrile. Extract the trapped benfuracarb from the adsorbent into acetonitrile by ultrasonic extraction for 10 min. Take a 10-mL aliquot of the acetonitrile extract and concentrate to near dryness by rotary evaporation. Dissolve the residue in 1 mL of acetonitrile.

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6.2 Cleanup

First cleanup: Transfer the carbon tetrachloride solution into a glass column packed with 5 g of Florisil saturated in carbon tetrachloride. Rinse the column, first with 2 mL of carbon tetrachloride and then with 35 mL of hexane-ethyl acetate (9 : 1, v/v). Elute benfuracarb with 45 mL of the same hexane-ethyl acetate solution. Concentrate the eluate to dryness by rotary evaporation at 35 °C and dissolve the residue in carbon tetrachloride.

Second cleanup: Transfer the above carbon tetrachloride solution into a glass column packed with 7 g of silica gel saturated in carbon tetrachloride. Rinse the column, first with 2 mL of carbon tetrachloride and then with 35 mL of hexane–ethyl acetate (17 : 3, v/v). Elute benfuracarb with 30 mL of the same hexane–ethyl acetate solution. Concentrate the eluate to near dryness by rotary evaporation and prepare the GC/HPLC-ready sample solution by dissolving the residue either in benzene for plant material or in acetonitrile for water and soil.

6.3 Determination

6.3.1 Plant material

Inject an aliquot of the GC-ready sample solution into the gas chromatograph.

Operating conditions

Gas chromatograph	Model 7-AG, Shimadzu
Sample injector	Solventless sample injector
Column	Flexible quartz capillary column, 0.2-mm i.d., 25-m length, coated with OV-101 (stationary phase)
Column temperature	265 °C
Injection port temperature	300 °C
Detector	Flame thermionic detector, FTD-8
Detector temperature	300 °C
Gas flow rates	Helium carrier gas, 1.5 mL min^{-1} Hydrogen, 4 mL min^{-1} Air, 150 mL min^{-1}
Injection volume	8 μL

6.3.2 Soil, water and air

Inject an aliquot of the HPLC-ready sample solution into the high-performance liquid chromatograph.

Operating conditions

High-performance liquid chromatograph	Model LC-3A, Shimadzu
Column	Stainless-steel column, 4.6-mm i.d., 250-mm length
Stationary phase	Zorbax ODS
Mobile phase	Acetonitrile–water (13 : 7, v/v)
Flow rate	1.0 mL min^{-1}
Column temperature	50 °C
Detection	UV detector at 280 nm
Injection volume	20 μL (soil and water), 50 μL (air)

7 Evaluation

7.1 Method

7.1.1 Plant material

Quantitation is performed by the calibration technique. Construct a new calibration curve with benfuracarb standard solutions (0.2, 0.4, 0.6, 0.8 and 1.0 μg mL^{-1} in acetone) for each set of analyses. The benfuracarb peak usually appears at a retention time around 6.0 min.

Plot the peak area against the injected amount of benfuracarb. The injection volume (8 μL) should be kept constant as the peak area varies with the injection volume in flame thermionic detection. Before each set of measurements, check the GC system by injecting more than one standard solution containing ca 5–10 ng of benfuracarb. Recommendation: inject standard solutions and sample solutions alternately rather than constructing the calibration curve in advance.

7.1.2 Soil, water and air

Quantitation is performed by the calibration technique. Construct a new calibration curve with benfuracarb standard solutions (2.0, 4.0, 6.0, 8.0 and 10.0 $\mu g\,mL^{-1}$ in acetonitrile) for each set of analyses. Inject 20-μL (50-μL for air) aliquots of the standard solutions. The retention time is around 9.8 min. Plot the peak area against the injected amount of benfuracarb.

7.2 Recoveries, limit of detection and limit of determination

7.2.1 Plant material

With fortification levels between 0.05 and 0.5 $mg\,kg^{-1}$, recoveries from untreated plant matrices ranged from 75 to 100% with the limit of determination being 0.005 $mg\,kg^{-1}$.

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7.2.2 Soil

With fortification levels between 0.1 and 1.0 $mg\,kg^{-1}$, recoveries from blank soils ranged from 77 to 92% with the limit of determination being 0.04 $mg\,kg^{-1}$.

7.2.3 Water

With fortification levels between 0.05 and 1.0 $mg\,kg^{-1}$, recoveries from blank water ranged from 95 to 98% with the limit of determination being 0.001 $mg\,kg^{-1}$.

7.2.4 Air

With fortification levels between 400 and 1000 ng in 100 mg of adsorbent, recoveries from the adsorbent ranged from 95 to 108% with the limit of determination being 0.010 $mg\,L^{-1}$.

7.3 Calculation of residues

The amount of benfuracarb residue (R, $mg\,kg^{-1}$) in the sample is calculated by the following equation:

$$R = C \times V/G$$

where

C = concentration of benfuracarb in the final solution ($\mu g\,mL^{-1}$)
V = final sample volume (mL)
G = original sample weight (g)

8 Important points

As suggested previously, the acid-labile N–S bond of the benfuracarb molecule should be protected throughout the homogenization and extraction procedures. Homogenization must be carried out in the presence of silver nitrate with the pH buffered at 7.0. During evaporation of organic solvents, the temperature of the water-bath should be kept at 35 °C or lower. Benfuracarb residue in the sample is stable under storage conditions at −20 °C for at least 80 days.

References

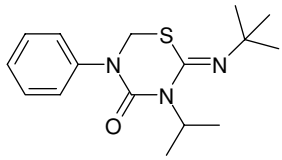
1. H. Mori, M. Kobayashi, K. Yagi, M. Takahashi, T. Gondo, and N. Umetsu, *J. Pestic. Sci.,* **12**, 491 (1987).
2. M. Kobayashi, K. Yagi, H. Mori, K. Endo, A. Tanaka, and N. Umetsu, *J. Pestic. Sci.,* **12**, 689 (1987).

Katsura Yagi and Noriharu Umetsu
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Buprofezin

Materials to be analyzed	Plants (rice, citrus, apple, pear, peach, apricot, grape, cotton, tea, tomato, cucumber, eggplant, pepper, squash), soil and water
Instrumentation	Gas-chromatographic determination [mass spectrometric detection (MSD) or nitrogen–phosphorus detection (NPD)] for plant materials, soil and water

1 Introduction

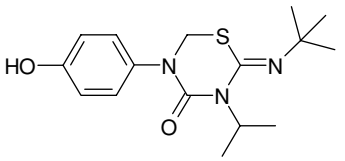
Chemical name (IUPAC)	2-*tert*-Butylimino-3-isopropyl-5-phenyl-1,3,5-thiadiazinan-4-one
Structural formula	
Empirical formula	$C_{16}H_{23}N_3OS$
Molar mass	305.4
Melting point	104.5–105.5°C
Vapor pressure	4.2×10^{-5} Pa at 20°C
Solubility (at 25°C)	Water (0.38 mg L^{-1}) n-Hexane (20 g L^{-1}) Chloroform (520 g L^{-1}) Acetone (240 g L^{-1}) Toluene (320 g L^{-1})
Stability	Stable in acidic and alkaline conditions Stable to heat and light
Other properties	Log K_{ow} (4.3)
Use pattern	Buprofezin is an insect growth regulator controlling homopterous insect pests, such as the brown rice planthopper[1] and the greenhouse whitefly.[2,3] Buprofezin inhibits chitin and prostaglandin synthesis[4] and has an insect hormone disturbing effect, leading to suppression of ecdysis.[5] Buprofezin shows insecticidal and acaricidal effects by inhibition of the moulting of nymphs and larvae

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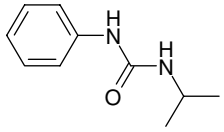
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with contact and stomach action; not translocated in the plant. Buprofezin suppresses oviposition by adults. Buprofezin-treated insects lay sterile eggs.

Regulatory position The major metabolite of buprofezin in plants is its *p*-hydroxy metabolite (*p*-OH-buprofezin) and that in soil is *1-isopropyl-3-phenylurea* (BF12). The target analytes are considered to be buprofezin and *p*-OH-buprofezin in plant materials, buprofezin and BF12 in soils and buprofezin in water samples.



p-OH-buprofezin

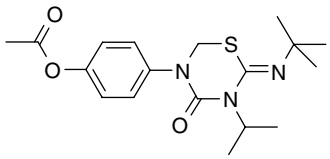


BF12

2 Outline of method

Buprofezin and its metabolites, *p*-OH-buprofezin and BF12, are hydrophobic under neutral conditions. Having the organic base part in their chemical structure, these compounds form water-soluble salts under strongly acidic conditions. The change in solubilities of these compounds influences the cleanup procedure.[6] Four different residue analytical methods have been developed to measure buprofezin and its metabolites in plants (rice, citrus and tomato; cucumber, pepper, tomato, squash and eggplant), soil and water:

A *'Multi-residue analytical method (for plants)'* is used for rice, citrus and tomato. Buprofezin and *p*-OH-buprofezin (as the acetylated derivative, *p*-AcO-buprofezin) are analyzed simultaneously using gas chromatography/nitrogen–phosphorus detection (GC/NPD).



p-AcO-buprofezin

B *'Gas chromatography/mass spectrometry (GC/MS) method (for plants)'* is used to determine buprofezin in cucumber, pepper, tomato, squash and eggplant.[7]

C *'Multi-residue analytical method (for soil)'* determines buprofezin and BF12 in soil sample simultaneously using GC/NPD.

D *'GC/MS method (for water)'* determines buprofezin in water samples.

3 Multi-residue analytical method (for plants)

3.1 Apparatus

Erlenmeyer flask, 500-mL
Round-bottom flask, 500-mL
Rotary evaporator
Separatory funnel, 200-mL
Fused-silica megabore column, DB-1, 15 m × 0.53-mm i.d., 1.5-μm film thickness, 100% dimethylpolysiloxane
Hewlett-Packard 5890A gas chromatograph with capillary split/splitless inlet with nitrogen–phosphorus detector equipped with a Model 7673A autosampler

3.2 Reagents and supplies

Acetone, reagent grade
Methanol, reagent grade
n-Hexane, reagent grade
1 N Hydrochloric acid (HCl), reagent grade
10 M Sodium hydroxide (NaOH), reagent grade
1 M Sodium phosphate buffer (pH 7), reagent grade
Pyridine, reagent grade
Acetic anhydride, reagent grade

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3.3 Procedure

3.3.1 Extraction

Weigh 50 g of the ground hulled rice, citrus flesh, or tomato sample (20 g citrus peel or rice straw) into a 500-mL Erlenmeyer flask and extract with 250 mL of acetone (or methanol for rice straw) by shaking for 1 h. Filter by suction and collect the extract in a 500-mL round-bottom flask. Wash the cake with 100 mL of acetone (or methanol for rice straw) and filter off. Combine the filtrates and concentrate to around 2 mL at 40 °C with a rotary evaporator.

3.3.2 Cleanup

Hexane solvent partitions

Transfer the concentrate into a 200-mL separatory funnel with 40 mL of n-hexane and 30 mL of 1 N HCl. After shaking for 5 min, drain the aqueous layer into another 200-mL separatory funnel and extract the n-hexane layer further with 30 mL of 1 N HCl. Combine the aqueous layers and neutralize to pH 7 with 6 mL of 10 M NaOH and 50 mL of 1 M phosphate buffer solution. Extract buprofezin and *p*-OH-buprofezin in the neutralized aqueous solution with 50 mL of n-hexane twice.

Combine the n-hexane layers, dry over anhydrous sodium sulfate, and concentrate to around 5 mL.

Derivatization

Add 0.2 mL of pyridine and 0.1 mL of acetic anhydride to the n-hexane solution for the acetylation of *p*-OH-buprofezin. Keep the mixture at ambient temperature overnight. Wash the reaction mixture with 10 mL of the distilled water twice and collect the n-hexane phase for GC/NPD analyses.

3.3.3 Determination

Inject an aliquot of the gas chromatography (GC)-ready sample solution into the GC/NPD system.

Operating conditions for GC/NPD

Gas chromatograph	Model 5890A, Hewlett-Packard
Sample injector	Splitless mode
Column	Fused-silica megabore column, DB-1, 15 m × 0.53-mm i.d., 1.5-μm film thickness
Column temperature	230 °C, constant
Injection port temperature	250 °C
Detector temperature	250 °C
Detector	Nitrogen–phosphorus detector
Gas flow rates	Helium carrier gas, 15 mL min^{-1} Hydrogen, 4 mL min^{-1} Air, 150 mL min^{-1}
Injection volume	1–2 μL

3.4 Evaluation

3.4.1 Method

Standardization

Peaks of buprofezin and *p*-AcO-buprofezin usually appear at retention times of around 1.7 and 4.6 min, respectively. Plot the peak areas against the amounts of the analytes.

Detection of sample residues

Inject the cleaned-up sample into the GC/NPD system operated under the same conditions as employed for standardization.

Compare the peak areas of the analytical samples with the calibration curve. Determine the concentration of buprofezin and *p*-AcO-buprofezin present in the sample.

p-OH-buprofezin residues are calculated from the following equation:

$$[p\text{-OH-buprofezin (mg kg}^{-1})] = [p\text{-AcO-buprofezin (mg kg}^{-1})] \times 0.884$$

where 0.884 is the molecular weight ratio of *p*-OH-buprofezin to *p*-AcO-buprofezin.

3.4.2 Recoveries, limit of detection and limit of determination

With a fortification level of 1–2 mg kg^{-1}, recoveries of buprofezin from untreated hulled rice, rice straw, tomato, citrus flesh and citrus peel samples are 85, 97, 93, 87 and 75%, respectively.

With a fortification level of 1–2 mg kg^{-1}, recoveries of *p*-OH-buprofezin from untreated hulled rice, rice straw, tomato, citrus flesh and citrus peel samples are 82, 82, 91, 95 and 90%, respectively.

The limits of detection of buprofezin and *p*-OH-buprofezin are 0.005 mg kg^{-1} for hulled rice, tomato and citrus flesh and 0.01 mg kg^{-1} for rice straw and citrus peel.

3.4.3 Calculation of residues

Calculate the concentrations of the analyte (buprofezin and *p*-OH-buprofezin) in plants (mg kg^{-1}) by the following equation:

$$\text{Analyte concentration} = \frac{A \times V}{W}$$

where

A = concentration of the analyte in the final solution (μg mL^{-1})
V = volume of the final solution (mL)
W = weight of analysis sample (g)

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3.5 Important points

The adjustment of the pH in the step Hexane Solvent Partitions to pH 7 should be checked.

4 GC/MS method (for plants)

4.1 Apparatus

High-speed blender
Buchner funnel, 12-cm
Rotary evaporator
ABC Laboratories Model SP 1000 gel permeation chromatograph system equipped with a 31.0 × 2.5-cm glass column of Envirobeads SX-3 select 200–400 mesh (ca 60 g) preconditioned with ethyl acetate–cyclohexane (1 : 1, v/v)
Hewlett-Packard 5890 gas chromatograph with capillary split/splitless inlet with HP5971 mass-selective detector equipped with a Model 7673 autosampler
Fused-silica capillary column, HP-Ultra 2, 25 m × 0.20-mm i.d., 0.33-μm film thickness, (5% phenyl)methylpolysiloxane

4.2 Reagents and supplies

Sodium sulfate, anhydrous, reagent grade
Ethyl acetate, reagent grade
Whatman ashless 40 filter paper
Cyclohexane, reagent grade

4.3 Procedure

4.3.1 Extraction

Weigh 50 g of the plant sample into a high-speed blender jar containing 50 g of anhydrous sodium sulfate. Mix thoroughly and add 150 mL of ethyl acetate to the mixture. After homogenization, filter the supernatant liquid through a layer of 20 g of anhydrous sodium sulfate on Whatman ashless 40 filter paper in a 12-cm Buchner funnel. After adding plant tissues to the funnel, repeat the extraction and filtration steps once more with 100 mL of ethyl acetate. Combine the extracts and concentrate to 2–3 mL at 40 °C with a rotary evaporator.

4.3.2 Cleanup

Gel permeation chromatography (GPC)

Adjust the final volume of concentrated sample (2–3 mL) to 5 mL with ethyl acetate and then to 10 mL with cyclohexane. Apply 5 mL of this sample extract to the gel permeation system and collect the elution volume fraction 110–140 mL. Evaporate this fraction to dryness at 40 °C with a rotary evaporator and dissolve the residue in 5 mL of cyclohexane for GC/MSD analysis.

4.3.3 Determination

Inject an aliquot of the GC-ready sample solution into the GC/MSD system.

Operating conditions for GC/MS

Gas chromatograph	Model 5890, Hewlett-Packard
Sample injector	Splitless mode
Column	Fused-silica capillary column, HP-Ultra 2, 25 m × 0.20-mm i.d., 0.33-μm film thickness
Column temperature	Initial 55 °C, held for 1 min; increased at 30 °C min^{-1} to 140 °C, held for 1 min; increased at 5 °C min^{-1} to 260 °C, held for 6 min
Injection port temperature	280 °C
Interface temperature	280 °C
Detector	Mass-selective detector, MSD5971
Selected ion monitoring	*m/z* 105, 172, 305
Gas flow rate	Helium carrier gas, 1 mL min^{-1}
Injection volume	5 μL

4.4 Evaluation

4.4.1 Method

Standardization

The peak of buprofezin usually appears at a retention time of around 25.5 min. Plot the log of peak area against the log of concentrations of the analytes.

Detection of sample residues

Inject the cleaned-up sample into the GC/MS system operated under the same conditions as employed for standardization.

Compare the peak areas of the analytical samples with the calibration curve. Determine the concentration of buprofezin present in the sample.

4.4.2 Recoveries, limit of detection and limit of determination

With fortification levels between 0.01 and 0.61 mg kg^{-1}, recoveries of buprofezin from untreated cucumber samples ranged from 73 to 86%.

With a fortification level of 0.20 mg kg^{-1}, recoveries of buprofezin from untreated pepper, tomato, squash and eggplant samples were 76, 73, 77 and 92%, respectively.

The limit of quantification of buprofezin is 0.01 mg kg^{-1}.

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4.4.3 Calculation of residues

Calculate the concentrations of buprofezin in plants (mg kg^{-1}) by the following equation:

$$\text{Buprofezin concentration} = \frac{A \times V}{W}$$

where

A = concentration of the analyte in the final solution (μg mL^{-1})
V = volume of the final solution (mL)
W = weight of analysis sample (g).

5 Multi-residue analytical method (for soil)

5.1 Apparatus

Erlenmeyer flask, 500-mL
Buchner funnel
Round-bottom flasks, 125- and 500-mL
Rotary evaporator

Separatory funnels, 125- and 250-mL
Disposable filtration column
Fused-silica megabore column, DB-1, 15 m × 0.53-mm i.d., 1.5-μm film thickness, 100% dimethylpolysiloxane
Hewlett-Packard 5890A gas chromatograph with capillary split/splitless inlet with nitrogen–phosphorus detector equipped with a Model 7673A autosampler

5.2 Reagents and supplies

Acetone, reagent grade
Whatman 934-AH filter paper
n-Hexane, reagent grade
1 N HCl, reagent grade
Dichloromethane, reagent grade
Sodium sulfate, anhydrous, reagent grade
Toluene, reagent grade

5.3 Procedure

5.3.1 Extraction

Weigh 50 g (dry weight) of the soil sample into a 500-mL Erlenmeyer flask and extract with 150 mL of acetone by shaking for 30 min. Filter the extracts by suction through a Buchner funnel using Whatman 934-AH filter paper and collect the extracts in a 500-mL round-bottom flask. Concentrate the organic extracts at 40 °C under reduced pressure.

5.3.2 Cleanup

Hexane solvent partition

Transfer the concentrate into a 125-mL separatory funnel with 5 mL of deionized water, 20 mL of n-hexane and 30 mL of 1 N HCl. After shaking, drain the aqueous layer into a 250-mL separatory funnel and extract the n-hexane layer further with 30 mL of 1 N HCl. Combine the aqueous layers.

Extract buprofezin and BF12 from the acidic aqueous phase with 25 mL of dichloromethane twice. Dry the extracts by passing them through anhydrous sodium sulfate held in a disposable filtration column and collect in a 125-mL round-bottom flask.

Rotary evaporate the extracts to dryness at 40 °C under reduced pressure and dissolve the residue in 5 mL of toluene for GC/NPD analyses.

5.3.3 Determination

Inject an aliquot of the GC-ready sample solution into the GC/NPD system.

Operating conditions for GC/NPD

Gas chromatograph	Model 5890, Hewlett-Packard
Sample injector	Splitless mode
Column	Fused-silica megabore column, DB-1, 15 m × 0.53-mm i.d., 1.5-μm film thickness
Column temperature	Initial 165 °C, held for 2 min; increased at 20 °C min^{-1} to 185 °C, held for 2 min; increased at 20 °C min^{-1} to 235 °C, held for 2.5 min
Injection port temperature	275 °C
Detector temperature	300 °C
Detector	Nitrogen–phosphorus detector
Gas flow rates	Helium carrier gas, 24 mL min^{-1} Hydrogen, 3.5 mL min^{-1} Air, 100 mL min^{-1}
Injection volume	1–2 μL

5.4 Evaluation

5.4.1 Method

Standardization

Peaks of BF12 and buprofezin usually appear at retention times around 3.8 and 8.5 min, respectively. Plot the peak areas against the concentrations of the analytes.

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Detection of sample residues

Inject the cleaned-up sample into the GC/NPD system operated under the same conditions as employed for standardization.

Compare the peak areas of the analytical samples with the calibration curve. Determine the concentration of buprofezin and BF12 present in the sample.

5.4.2 Recoveries, limit of detection and limit of determination

With fortification levels between 0.01 and 1.00 mg kg^{-1}, recoveries of buprofezin from untreated soil samples ranged from 87 to 90% with the limit of determination being 0.01 mg kg^{-1}.

With fortification levels between 0.01 and 1.00 mg kg^{-1}, recoveries of BF12 from untreated soil samples ranged from 90 to 97% with the limit of determination being 0.01 mg kg^{-1}.

5.4.3 Calculation of residues

Calculate the concentrations of the analytes (buprofezin and BF12) in soil samples (mg kg^{-1}) by the following equation:

$$\text{Analyte concentration} = \frac{A \times V}{W}$$

where

A = concentration of the analyte in the final solution ($\mu g\,mL^{-1}$)
V = volume of the final solution (mL)
W = weight of analysis sample (g)

6 GC/MS method (for water)

6.1 Apparatus

Rotary evaporator
Fused-silica capillary column, XTI-5, 30 m × 0.25-mm i.d., 0.25-μm film thickness, (5% phenyl)methylpolysiloxane
Hewlett-Packard 5890 gas chromatograph with capillary split/splitless inlet with HP5971 mass-selective detector equipped with a Model 7673 autosampler

6.2 Reagents and supplies

Dichloromethane, reagent grade
Sodium sulfate, anhydrous, reagent grade
Ethyl acetate, reagent grade

6.3 Procedure

6.3.1 Extraction

Extract the sample of water (1000 mL) three times with 50 mL of dichloromethane. Dry with anhydrous sodium sulfate and remove the combined organic phase by rotary evaporation. Dissolve the remaining residue with 1 mL of ethyl acetate for GC/MSD analysis.

6.3.2 Cleanup

This step is not required for water samples.

6.3.3 Determination

Inject an aliquot of the GC-ready sample solution into the GC/MSD system.

Operating conditions for GC/MS

Gas chromatograph	Model 5890, Hewlett-Packard
Sample injector	Splitless mode
Column	Fused-silica capillary column, XTI-5, 30 m × 0.25-mm i.d., 0.25-μm film thickness

Gas flow rate	Helium carrier gas, 1 mL min^{-1}
Column temperature	Initial 60 °C, held for 1 min, increased at 25 °C min^{-1} to 250 °C, held for 7 min
Injection port temperature	250 °C
Interface temperature	280 °C
Detector	Mass-selective detector, MSD5971
Selected ion monitoring	*m/z* 172 (quantification) *m/z* 105, 175, 305 (verification)
Injection volume	1 μL

6.4 Evaluation

6.4.1 Method

Standardization

The peak of buprofezin usually appears at a retention time around 11.4 min. Plot the log of peak area against the log of concentration of the analyte.

Detection of sample residues

Inject the cleaned-up sample into the GC/MS system operated under the same conditions as employed for standardization.

Compare the peak areas of the analytical samples with the calibration curve. Determine the concentration of buprofezin present in the sample.

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6.4.2 Recoveries, limit of detection and limit of determination

With fortification levels between 0.050 and 5.0 μg L^{-1}, recoveries of buprofezin from untreated water samples ranged from 103 to 110% with the limit of determination being 0.05 μg L^{-1}.

6.4.3 Calculation of residues

Calculate the concentrations of buprofezin in water samples (μg L^{-1}) by the following equation:

$$\text{Buprofezin concentration} = \frac{A \times V}{W}$$

where

A = concentration of the analyte in the final solution (μg mL^{-1})
V = volume of the final solution (mL)
W = volume of analysis sample (mL).

References

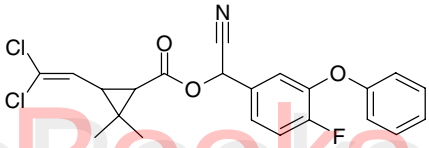
1. H. Kanno, K. Ikeda, T. Asai, and S. Maekawa, in 'Proceedings of the 1981 British Crop Protection Conference on Pests and Disease,' Vol. 1, 59–66 (1981).
2. M. Yasui, M. Fukada, and S. Maekawa, *Appl. Entomol. Zool.,* **20**, 340 (1985).
3. M. Yasui, T. Nishimatsu, M. Fukada, and S. Maekawa, *Appl. Entomol. Zool.,* **26**, 271 (1991).
4. M. Uchida, Y. Izawa, and T. Sugimoto, *Pestic. Biochem. Physiol.,* **27**, 71 (1989).
5. M. Kobayashi, M. Uchida, and K. Kuriyama, *Pestic. Biochem. Physiol.,* **34**, 9 (1989).
6. H. Nishizawa, M. Shigemura, T. Suzuki, and M. Uchida, *J. AOAC Int.,* **77**, 1631 (1994).
7. A. Valverde-García, A.R. Fernández-Alba, J.C. Herrera, and E. Roldán, *J. AOAC Int.,* **77**, 1041 (1994).

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Cyfluthrin

Materials to be analyzed	Apple, pears, wheat forage, cantaloupe, cucumbers, squash, lettuce, mustard greens, plums, peaches, cherries, peanuts, potatoes, tomatoes, peppers, grapes, radish, carrots, tobacco, oranges, lemons, grapefruits, celery, spinach, cabbage
Instrumentation	Gas chromatography/mass spectrometry (GC/MS)

1 Introduction

Chemical name (IUPAC)	Cyano(4-fluoro-3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate
CAS No.	68359-37-5
Structural formula	
Empirical formula	$C_{22}H_{18}Cl_2FNO_3$
Molecular weight	434.3
Melting point	First melt, 71.9 °C; second melt, 86–95 °C
Physical form	Amber semi-solid gel
Vapor pressure	3.3×10^{-8} mmHg at 20 °C 6.3×10^{-8} mmHg at 30 °C 2.3×10^{-7} mmHg at 40 °C
Solubility	Water (at pH 3 and 20 °C): Isomer 1, 2.5 μg L^{-1} Isomer 2, 2.1 μg L^{-1} Isomer 3, 3.2 μg L^{-1} Isomer 4, 4.3 μg L^{-1} Water (at pH 7 and 20 °C): Isomer 1, 2.2 μg L^{-1} Isomer 2, 1.9 μg L^{-1} Isomer 3, 2.2 μg L^{-1} Isomer 4, 2.9 μg L^{-1} Highly soluble in dioxane, methylene chloride, toluene

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Use pattern	Cyfluthrin is a broad-spectrum pyrethroid insecticide which provides general insect control in agricultural crops, in food-handling establishments, in the garden, and in/around the home.
Regulatory position	The residue definition consists of cyfluthrin alone.

2 Outline of method

Macerated plant material is homogenized with a mixture of methanol and 1.2 N hydrochloric acid (HCl) in water (4 : 1, v/v) and then with methanol. An internal standard solution is added to the filtrate and the filtrate is adjusted to a constant volume. A portion of the filtrate is rotary evaporated to dryness and hexane is added to the extract before a Florisil cleanup procedure is performed. The extract is dissolved in toluene for analysis by GC/MS in the negative chemical ionization (NCI) mode.

3 Apparatus

Assorted laboratory supplies (including, but not limited to):
- Beakers, tall-form, stainless steel, 300-mL or equivalent
- Buchner funnels, 9-cm
- Cylinders, graduated, 100-mL
- Flasks, round-bottom with 24/40 ground-glass joint, 500-mL
- Flasks, volumetric, Class A, various sizes
- Funnels, glass
- Kontes columns, bell-shaped, 15-mm i.d., 250-mm length, 250-mL reservoir
- Pipets, volumetric, Class A, various sizes
- Spatulas, stainless steel

Balances:
- Top-loader electronic, accurate to 0.0001 g
- Top-loader electronic, accurate to 0.01 g

Column, gas chromatograph: HP-5, 5% phenyl–methyl capillary column, 0.25-mm i.d. × 12-m length, 0.33-μm film thickness (Agilent Technologies) or equivalent
- Evaporator, rotary vacuum, water-bath set at 60 and 40 °C

Processors:
- Disk mill
- Vertical batch processor

Gas chromatograph, Model 6890 (Agilent Technology) equipped with split/splitless injector and a mass spectrometer, Model 5973, or equivalent

Tissumizer, Model SDT 1810 S1 with S25N probe (Tekmar, Cincinnati, OH, USA) or equivalent

4 Reagents/supplies and reference standards

4.1 Reagents/supplies

Bags, sample storage, plastic
Bottles:
  Round, flint glass with polymer-lined lids, 120-mL
  Square, flint glass with polymer-lined lids, 0.5-oz
Filter cartridges, Acrodisc nylon, 25-mm (Gelman Sciences, Ann Arbor, MI, USA) or equivalent
Filter, GF/A, 9-cm
Florisil, 2.5% deactivated
Glass wool
Sodium sulfate, anhydrous, ACS grade (Fisher Scientific) or equivalent
Solvents: acetonitrile (ACN), acetone, hexane, methanol (MeOH) and water (pesticide or HPLC grade)
Gases, compressed (purity ≥99%), methane, helium
Syringes, plastic, single-use, 3-mL
Vials: autosampler, clear glass with Teflon-lined septum caps, 1.8-mL

4.2 Reference materials

Cyfluthrin (provided by Bayer Corp.): cyano(4-fluoro-3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate, molar mass 434.3, ion observed at 207.0 (GC/MS)

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Cyfluthrin-*methyl*-d_6 (provided in acetonitrile solution by Bayer Corp.): [^3H]-Cyfluthrin stable-isotope internal standard (IS), Cyclopropanecarboxylic acid, 3-(2,2-dichloroethenyl)-2,2-dimethyl-, cyano(4-fluoro-3-Phenoxyphenyl)methyl ester-*methyl*-d_6, molecular formula: $C_{22}H_{12}D_6Cl_2FNO_3$, molar mass 440.3, ion observed at 213.0 (GC/MS)

5 Sampling and preparation

Plant material should be added to a disk mill (grain or seed matrices) or a vertical batch processor (all other matrices). Add an equal portion of pelletized dry ice to the sample (vertical processor only). Macerate the plant sample (or sample + dry ice) until a homogeneous mixture is obtained. Store the homogenized mixture in doubled plastic bags at $-20 \pm 5\,^{\circ}C$. For samples containing dry ice, allow the open bag to stand in the freezer overnight to allow the dry ice to sublime prior to sealing the bag.

6 Procedures

6.1 Extraction

Weigh 5.0 g of frozen homogenized sample into a 120-mL tall-form glass bottle. Begin recovery samples at this point by fortifying the control tissue matrix samples.

Add 45 mL of methanol–1.2 N HCl in water (4 : 1, v/v) to the sample, and blend this mixture with a Tissumizer fitted with an S25N mixing probe for 2 min. Transfer the contents of the bottle into a 9-cm Buchner funnel containing a GF/A filter, and vacuum filter the extract into a 100-mL graduated mixing cylinder. Return the filter cake and the filter paper to the bottle, add 45 mL of methanol to the bottle, and homogenize the contents of the bottle for 2 min using the same Tissumizer. Filter the methanol into the same graduated cylinder. Maintain vacuum until the filter cake is dry, and discard the filter cake. Add 100 μL of a 2.5 μg mL^{-1} deuterated internal standard solution, dissolved in acetonitrile, to the cylinder. Adjust the filtrate volume to 100 mL with methanol, stopper the graduated cylinder and mix the contents.

Measure 50 mL of the filtrate into a 250-mL boiling flask and evaporate the filtrate aliquot to dryness under vacuum using a water-bath at 60 °C. Remove the boiling flask from the evaporator, add 25 mL of hexane to the flask, and sonicate the contents in a water-bath for 1 min.

6.2 Cleanup

Prepare a Florisil column in the following manner: close the drain valve of a bell-shaped Kontes column, place glass wool at the bottom and add approximately 100 mL of hexane to the column. Weigh 7 g of 2.5% deactivated Florisil, place the Florisil in the column, and tap the column gently until the Florisil settles to the bottom of the column. Add 6 g of anhydrous sodium sulfate to the column, and drain the hexane into a waste container until the hexane is just above the sodium sulfate layer. Transfer 25 mL of sample extract from the boiling flask into the column and let the extract run dropwise through the column into a waste container. Add 40 mL of additional hexane to the boiling flask, swirl the contents to mix, pour the flask contents into the column, and let the hexane run through the column into a waste container.

Elute cyfluthrin with 60 mL of hexane–acetone (9 : 1, v/v) dropwise into a 125-mL boiling flask. Evaporate the eluted sample to dryness under vacuum in a water-bath at 40 °C. Pipet 2.5 mL of toluene into the boiling flask and rotate the flask to dissolve all residues. Filter approximately 1 mL of the dissolved residue through a 25-mm Acrodisc nylon filter cartridge into an autosampler vial.

6.3 Determination

Gas chromatograph conditions

Instrument	Hewlett-Packard 6890 gas chromatograph with split/splitless injector
Column	HP-5, 5% phenyl–methyl capillary column, 0.25-mm i.d. × 12-m length, 0.33-μm film thickness
Injection port	Splitless mode Temperature 250 °C
Gas	Methane (carrier gas)
Injection volume	1 μL

Column program	150 °C from 0 to 4 min, increased at 30 °C min^{-1} to 300 °C, held 11 min
Retention time	9 min (approximate)
Total run time	20 min

Mass spectrometer conditions

Instrument	Hewlett-Packard 5973 mass spectrometer
Acquisition mode	Selected ion monitoring (SIM)
Ionization mode	NCI
MS quadrupole	Temperature 106 °C
MS source	Temperature 150 °C
MSD transfer line heater	Temperature 300 °C
Ion masses	207.0 for cyfluthrin 213.0 for cyfluthrin-*methyl*-d_6
Solvent delay	4 min

7 Evaluation

7.1 Method

Since the internal standard (IS) solution is added to the sample at a point just after extraction, no addition of IS to the final sample extract is required. An external calibration standard solution with a 0.05 mg L^{-1} concentration of both native and internal standard cyfluthrin, dissolved in toluene, is utilized to bracket a series of 1–10 injections of various sample extracts. Calculation of cyfluthrin concentration in the final sample extract is made by comparison of the response ratio (cyfluthrin/deuterated cyfluthrin) in the sample to the averaged response ratios found in the external calibration solution injections that bracket the samples.

Instrumental response ratios (cyfluthrin/deuterated cyfluthrin) versus concentration of cyfluthrin present should be proven in solvent and each matrix analyzed up to the highest undiluted final sample extract concentration expected.

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7.2 Recoveries, limits of detections, and limits of quantitation

Net recoveries of cyfluthrin from matrices fortified at 0.01–5.05 mg kg^{-1} ranged from 77 to 119%. The limit of detection (LOD) is defined as the lowest concentration that can be determined to be statistically different from a blank or control. Calculate the value by taking the standard deviation of the residue values from the analysis of the recovery samples at the limit of quantification (LOQ) and using the equation

$$\text{LOD} = \text{standard deviation} \times t_{0.99}$$

where $t_{0.99}$ = the one tailed t-statistic at the 99% confidence level for $n - 1$ replicates.

The LOD ranged from 0.0005 to 0.004 mg kg^{-1}. The LOQ based on the lowest concentration level fortified of cyfluthrin was 0.01 mg kg^{-1} for all the plant matrices analyzed.

7.3 Calculation of residues

The amount of cyfluthrin residue (R, mg kg^{-1}) in the sample is calculated by the following equation:

$$R = (\text{sample response ratio})/(\text{av. standard response ratio}) \times \text{standard concentration}$$

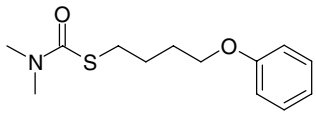
where standard concentration = mg kg^{-1} cyfluthrin equivalent in the final sample extract (i.e., 0.05 mg L^{-1}).

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Fenothiocarb

Materials to be analyzed	Mandarin oranges (juice, pulp, rind), leaves of mandarin orange tree, soil
Instrumentation	Gas-chromatographic determination

1 Introduction

Chemical name (IUPAC)	(*S*)-4-Phenoxybutyl dimethylthiocarbamate
Structural formula	
Empirical formula	$C_{13}H_{19}NO_2S$
Molar mass	253.4
Melting point	40–41 °C
Boiling point	155 °C at 0.02 mmHg
Vapor pressure	0.166 mPa at 23 °C
Solubility	In water 30 mg L^{-1} (20 °C). In cyclohexanone 3800, acetonitrile 3120, acetone 2530, xylene 2464, methanol 1426, kerosene 80, hexane 66 g L^{-1} (20 °C).
Stability	Slowly decomposed by sunlight. Stable to hydrolysis for 5 days (pH 5–9, 40 °C); <1% decomposition after 60 days at 55 °C.
Use pattern	Fenothiocarb is a nonsystemic acaricide used to control the eggs and larval stages of *Panonychus* spp.
Regulatory position	The residue definition is for the parent, fenothiocarb only.

DropBooks

2 Outline of method

Plant substances (juice, pulp, rind, and leaf) are homogenized with anhydrous sodium sulfate and methanol and fenothiocarb residue is extracted with acidic methanol [0.5 N hydrochloric acid–methanol (1 : 3, v/v)] by refluxing for soil. Fenothiocarb in the

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extract of plant substances is extracted with hexane. Since oily plant substances affect the subsequent cleanup process, fenothiocarb in the hexane extract is partitioned into acetonitrile. The concentrated acetonitrile extracts are purified by silica gel column chromatography with benzene. Fenothiocarb in the soil extract is dissolved in dichloromethane and purified by silica gel column chromatography with benzene. Fenothiocarb is determined by gas chromatography using a sulfur-specific flame photometric detector.

3 Apparatus

Blender (kitchen type)
Juicer mixer (automatic juicer)
High-speed blender (Waring blender, or equivalent)
Bell jar-type filtering apparatus
Buchner funnel, 11-cm i.d.
Rotary vacuum evaporator, 40 °C bath temperature
Water-bath, electrically heated, temperature 70 °C
Condenser
Round-bottom flasks, 500- and 300-mL
Separatory funnels, 1000-, 300-, and 200-mL
Glass funnel, 10-cm i.d.
Glass chromatography column, 1.5-cm i.d. × 30 cm with a stopcock
Silica gel column: place a cotton wool plug at the bottom of a glass chromatography column. Pack 10 g of silica gel slurried with benzene into the glass column. Make an anhydrous sodium sulfate layer about 1-cm above and below the silica gel bed.
Gas chromatograph equipped with a sulfur-specific flame photometric detector
Microsyringe, 10-μL

4 Reagents

Acetone, acetonitrile, benzene, dichloromethane, n-hexane, and methanol, pesticide residue analysis grade
Distilled water, HPLC grade
Anhydrous sodium sulfate, hydrochloric acid (36%), sodium chloride, special grade
Silica gel, Wakogel C-100, adjust water content to 6.5% with distilled water (Wako Pure Chemical Industries, Ltd)
Filter aid, Celite 545 (Johns-Manville Products Corporation)
Filter paper, 11-cm i.d.
Fenothiocarb, analytical grade (Ihara Chemical Industries Co., Ltd)
Fenothiocarb standard solutions: 0.2, 0.5, 1, 2, and 3 $\mu g\,mL^{-1}$ in acetone

5 Sampling and sample preparation

Collect 2 kg of orange fruits randomly and use 1 kg for juice and the other 1 kg for pulp and rind. Prepare the juice by crushing oranges directly with a juicer mixer.

Homogenize pulp with a blender and rind by chopping with a knife. Collect 50 g of leaf randomly and homogenize by chopping with a knife. Soil, collected from the top10-cm surface layer, is homogenized and passed through a 5-mm sieve.

6 Procedure

6.1 Extraction

6.1.1 Orange juice and pulp

Homogenize 50 g of the sample for 5 min with 75 mL of methanol and 10 g of anhydrous sodium sulfate. Add 10 g of Cellite-545 to the homogenate and mix well, and filter the mixture through a filter paper in a Buchner funnel into a 300-mL round-bottom flask. Rinse the residue in a blender with 50 mL of a mixture of water and methanol (1 : 1, v/v). Combine the filtrates.

6.1.2 Orange rind

Homogenize 25 g of the sample for 5 min with 75 mL of methanol, 25 mL of water and 10 g of anhydrous sodium sulfate. Conduct the subsequent procedures in a similar manner as described for juice and pulp.

6.1.3 Orange leaves

Homogenize 5 g of the sample for 5 min with 45 mL of methanol, 25 mL of water and 5 g of anhydrous sodium sulfate. Conduct the subsequent procedures in a similar manner as described for juice and pulp. Rinse the residue with 30 mL of a mixture of water and methanol (1 : 1, v/v).

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6.1.4 Soil

Weigh 40 g (dry soil weight) of the sample into a 300-mL round-bottom flask, add 120 mL of acid–methanol solution [a mixture of 0.5 N hydrochloric acid and methanol (1 : 3, v/v)], and reflux the sample at 70 °C for 4 h after attaching a condenser.

Add 15 g of Cellite-545 to the sample and mix well, then filter the mixture through a filter paper in a Buchner funnel into a 500-mL flask. Rinse the 300-mL round-bottom flask and the residue with 100 mL of methanol.

6.2 Cleanup

6.2.1 Orange juice, pulp and rind

Transfer the sample extract (from Section 6.1) into a 300-mL separatory funnel, add 50 mL of water and extract the sample with 50 mL of n-hexane three times. Separate and dry the n-hexane layer with anhydrous sodium sulfate (plug the funnel with absorbent cotton and 50 g of anhydrous sodium sulfate), and collect the dried extract in a 300-mL of separatory funnel. Add 50 mL of acetonitrile to the separatory funnel and mix well for partitioning with the n-hexane extract three times. Collect the

acetonitrile layer in a 300-mL round-bottom flask. Evaporate the acetonitrile under reduced pressure. Dissolve the residue in 3 mL of benzene.

Adsorb the benzene solution on the top of the silica gel column (10 g of silica gel) and elute with benzene. Discard the first 50 mL of eluate. Collect the subsequent fractions of 150 mL in a 300-mL round-bottom flask and evaporate the solvent under reduced pressure. Dissolve the residue in an appropriate volume of acetone for analysis.

6.2.2 Orange leaves

Transfer the sample extract (from Section 6.1) into a 200-mL separatory funnel, add 30 mL of water and extract the sample extract three times with 30 mL of n-hexane. Collect and dry the n-hexane layer with anhydrous sodium sulfate in a funnel in a similar manner as described for the juice, pulp and rind, and evaporate the solvent under reduced pressure. Dissolve the residue in 3 mL of benzene and clean up the sample by silica gel column chromatography in a similar manner as described for juice, pulp and rind.

6.2.3 Soil

Transfer the soil extract (from Section 6.1) into a 1000-mL separatory funnel, add 200 mL of water and 10 mL of saturated sodium chloride solution, and extract the sample with 100 mL of dichloromethane three times. Dry the dichloromethane extract with anhydrous sodium sulfate in a funnel in a similar manner as described for juice, pulp and rind, and collect the dried solution in a 500-mL round-bottom flask. Evaporate the dichloromethane under reduced pressure. Dissolve the residue in 3 mL of benzene.

Cleanup the sample by silica gel column chromatography in a similar manner as described for juice, pulp and rind.

6.3 Gas-chromatographic determination

Inject an aliquot (V_i) of the solution prepared from Section 6.2 (V_{End}) into the gas chromatograph

Operating conditions	Gas chromatograph (Hitachi 163)
Column	Glass, 3-mm i.d. × 1.0-m length, packed with 1% FFAP on Chromosorb W HP, 100–120 mesh
Column temperature	215 °C
Injection port temperature	240 °C
Detector	Flame photometric detector fitted with a 394-nm sulfur-specific filter, temperature 150 °C
Gas flow rates	Nitrogen carrier gas, 50 mL min^{-1} Hydrogen, 50 mL min^{-1} Oxygen, 15 mL min^{-1}
Attenuation	32 × 100
Chart speed	10 mm min^{-1}

Injection volume	1–4 μL
Retention time	3.4 min
Minimum detectable amount	0.2 ng

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. Construct a fresh calibration curve with fenothiocarb standard solutions for each set of analyses.

Inject 1 μL of each fenothiocarb standard solution into the gas chromatograph. Using log–log paper, plot the peak heights in millimeters against the injected amount of fenothiocarb in nanograms.

Also inject 1–4-μL aliquots of the sample solutions. From the peak heights of the peaks obtained for these solutions, read the appropriate amounts of fenothiocarb from the calibration curve.

7.2 Recoveries, limit of detection and limit of determination

7.2.1 Edible part (juice, pulp)

The recoveries from control samples fortified with fenothiocarb at levels of 0.002–0.10 mg kg^{-1} ranged from 81 to 106% and from 87 to 90%, respectively.

The limit of detection was 0.001 mg kg^{-1} and the limit of determination was 0.002 mg kg^{-1}.

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7.2.2 Inedible part (rind, leaf)

The recoveries from control samples fortified with fenothiocarb at levels of 0.02–1.0 mg kg^{-1} ranged from 88 to 98% and from 80 to 94%, respectively.

The limit of detection was 0.01 mg kg^{-1} and the limit of determination was 0.02 mg kg^{-1}.

7.2.3 Soil

The recoveries from control samples fortified with fenothiocarb at levels of 0.2–0.4 mg kg^{-1} ranged from 86 to 100% and from 83 to 94%, respectively.

The limit of detection was 0.05 mg kg^{-1}.

7.3 Calculation of residues

The residue R, expressed in mg kg^{-1} fenothiocarb, is calculated from the following equation:

$$R = (W_A \times V_{End})/(V_i \times G)$$

where

G = sample weight (g)
V_{End} = terminal volume of sample solution from Section 6.2 (mL)
V_i = portion of volume V_{End} injected into gas chromatograph (μL)
W_A = amount of fenochiocarb for V_i read from calibration curve (ng)

8 Important points

1. Solvent for extraction of fenothiocarb in plant substances. Based on the examination using the field sample, methanol was selected as the solvent for extracting fenothiocarb in orange. The extraction efficiency of fenothiocarb was higher with acetone or methanol and was slightly lower with acetonitrile and dichloromethane. For the ability to extract fenothiocarb and metabolites[1] simultaneously,[2] methanol was superior to acetone.
2. Extraction method of fenothiocarb in soil.[3] The extraction efficient of fenothiocarb in soil was evaluated using diluvial soil (orange field) and volcanic ash soil allowed to stand in a greenhouse immediately after and for 5 days and 2 months under an upland field condition after addition of fenothiocarb at a level of 10 mg kg^{-1}. The extraction recovery of fenothiocarb in the sample immediately and 5 days after addition showed no marked difference but showed a substantial difference after 2 months, suggesting the extraction of fenothiocarb remaining in soil for a long time. The optimum time of refluxing for extraction showing the highest recovery of fenothiocarb was about 1 h for diluvial soil and about 4 h for volcanic ash soil.
3. Water used to adjust water content in silica gel should be sprayed uniformly. The water content is measured by heating at 105 °C. If it is not uniformly sprayed, the position of the first elution of fenothiocarb hardly changes, but the fraction will be collected over an extended period.
4. A large amount of oily substance in the plant extract can vary the elution volume of fenothiocarb in silica gel column chromatography.
5. Packing materials for column chromatography. Adequate cleanup can be achieved with alumina instead of silica gel. Activated carbon is not suitable for sample cleanup of ripe orange and leaf.
6. Sample storage stability. The level of fenothiocarb (0.4 mg kg^{-1}) in soils stored in the dark at 3 °C decreased to 94–97% after 40 days and to 68–82% after 120 days.

References

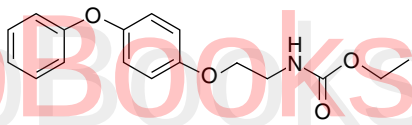
1. T. Unai, M. Tamaru, and C. Tomizawa, *J. Pestic. Sci.,* **11**, 347 (1986).
2. Y. Fukai, T. Oishi, Y. Asano and K. Ishikawa, 'An analytical method of fenothiocarb and its 3 metabolites in mandarin oranges (juice, pulp, rind),' in "Abstracts of the 9th Annual Meeting of the Pesticide Science Society of Japan," p. 150 (1984).
3. A. Yagi, Y. Asano, and K. Ishikawa, 'Extraction method for fenothiocarb in soils,' in "Abstracts of the 5th Annual Meeting of the Pesticide Residue Analysis Society," pp. 35–38 (1981) (in Japanese).

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Fenoxycarb

Materials to be analyzed	Air, water, soil, and plant (pasture grass hay, forage, cucurbits, citrus, pome fruit, tree nuts, fruiting vegetables, and cotton) and animal materials (tissues, milk, blood, and eggs)
Instrumentation	Liquid chromatography/ultraviolet detection, three-column switching liquid chromatography with fluorescence detection, two-column switching liquid chromatography/ultraviolet detection, gas chromatography/thermionic specific detection, gas chromatography/mass spectrometry, and liquid chromatography/atmospheric pressure ionization/mass spectrometry.

1 Introduction

Chemical name (IUPAC)	Carbamic acid, [2-(4-phenoxyphenoxy)ethyl]-, ethyl ester
Structural formula	
Empirical formula	$C_{17}H_{19}NO_4$
Molar mass	301.34
Melting point	53.6 °C
Vapor pressure	6.5×10^{-9} mbar at 25 °C
Solubility (25° C)	Soluble in water: 5.66 mg L^{-1} Readily soluble in organic solvents: ethanol 51, acetone 77, toluene 63, n-octanol 13, n-hexane 0.53 g per 100 mL
Other properties	Colorless to white solidified melt, no dissociation constant in an accessible pH range, octanol/water partition coefficient (log K_{ow}) 4.07 at 25 °C.
Use pattern	An insect growth regulator, used to control early instar larvae of Homoptera, Lepidoptera, and Coleoptera in citrus, cotton, and vines and fruiting vegetables
Regulatory position	The residue of concern is for the parent, fenoxycarb, only

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2 Outline of methods[1]

Air was sampled for a specific rate and time and the analyte collected on XAD-2 resin. The analyte was eluted from the resin using methanol followed by concentration of the eluate and analysis using gas chromatography/nitrogen–phosphorus detection (GC/NPD). Water was extracted by passing the sample through XAD-2 resin followed by elution of the analyte using ethyl acetate. Further purification of the extract was obtained using Florisil chromatography and final analysis was obtained using liquid chromatography/ultraviolet detection (LC/UV). Pond water was partitioned into hexane followed by evaporation of the solvent and analysis using LC/UV. Soil was extracted with acetone, acetonitrile was added, and the mixture partitioned with hexane. After discarding the hexane, the acetone–acetonitrile was adjusted to basic pH and re-partitioned with hexane. The hexane fraction was reduced and subjected to analysis using gas chromatography (GC)/thermionic specific detection. Plant and animal samples were extracted with acetone, filtered, and partitioned with hexane. After discarding the hexane, the acetone–acetonitrile fraction was adjusted to basic pH and re-partitioned with hexane followed by further purification using silica and Florisil SPE cartridges. Final analysis was accomplished using gas chromatography/mass spectrometry (GC/MS). Certain citrus samples were blended with C_8 or C_{18} SPE packing material and the mixture was loaded into a glass column. The analyte was eluted using dichloromethane–acetonitrile for final analysis using liquid chromatography/atmospheric pressure ionization/mass spectrometry (LC/API/MS).

3 Apparatus

3.1 Air

Air sampler, Alpha-1 (Messgerate-Werk Lauda, RMT 20, Germany)
Circulation cooler
Mini-Buck calibrator, Model M-5 (A.P. Buck, Inc.)
OSHA versatile sampler (OVS) sorbent tubes (SKC, Inc., Cat. No. 226-30-16)
Teflon vacuum pump (Analytichem International, N726.3FT.18)
Ultrasonic bath (Branson, Model 2200)

3.2 Water

Carbon filter tube (Fisher 08-261B)
Chromatography columns: 100 × 15-mm i.d. with Teflon stopcock
Glass-fiber filter (Whatman GF/D, 11-cm)
Rotary evaporator (Büchii) with water-bath temperature 50 °C

3.3 Soil

Buchner funnels, 7-cm
Evaporation flasks, 250- and 500-mL

Glass-microfiber filters, 7-mm (Whatman GF/D)
Reciprocating shaker
Separatory funnels, 125- and 250-mL
Side-arm flask, 500-mL
Wide-mouthed bottles with Teflon-lined lids, 250-mL

3.4 Pasture grass hay, forage, cucurbits, citrus, pome fruit, tree nuts, fruiting vegetables, and cotton substrates

Bottles, 8-oz
Round-bottom flasks, 50-, 100-, 250- and 1000-mL
Side-arm flasks, 500-mL
Florisil, 1000-g/6-cm^3 (J.T. Baker, 7213-07)
Homogenizer, Polytron (or equivalent)
PrepSep Florisil (Fisher Scientific, Cat. No. P476)
PrepSep Silica (Fisher Scientific, Cat. No. P478)
Silica, 100-g/6-cm^3 (J.T. Baker, 7086-07)
Sep-Pak, C_{18} (Waters, 43365)

3.5 Animal tissues, milk, blood, and eggs

Acrodisc filter, liquid chromatography (LC), poly(vinylidene fluoride) (PVDF), 0.2-µm, 13-mm
Bottles, 32-oz, wide-mouthed
Side-arm flasks, 500-mL
Flat-bottom boiling flasks, 250-mL
Glass-wool (Fisher, 11-390)
Silica gel solid-phase-extraction (SPE) cartridge, 1-g/6-cm^3 (J.T. Baker, 7086-07)
Other items as in Soil and Plant material lists

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4 Reagents

Acetone, glass distilled
Acetonitrile, LC grade
Ammonium hydroxide, ACS reagent grade
Anhydrous sodium sulfate, ACS reagent grade
Dichloromethane, HPLC grade
Ethyl acetate, glass distilled
Florisil (Fluka), 60–100-mesh
Hexane, glass distilled and residue analysis grade
Methanol, glass distilled and analytical grade
Phosphoric acid, ACS grade
Potassium dihydrogenphosphate, analytical grade
Potassium phosphate, monobasic, ACS grade

Sea sand, purified with acid and calcined
Sodium carbonate, ACS grade
Sodium sulfate, ACS grade
Toluene, high-purity
Water, glass distilled and LC grade
XAD-2 resin (0.15–0.20-mm particle size), research grade

5 Sample preparation

5.1 Air

Air was sampled by passing air at 0.5 L min^{-1} for 4 h (via vacuum) through an OVS tube containing a glass-fiber filter (to trap aerosols and particulates) and XAD-2 resin (to trap vapors). A second XAD-2 section in the sampling tube provided a means of checking for overloading of the first XAD-2 section. After sampling, the glass-fiber filter and first section of XAD-2 resin were transferred to a 10-mL round-bottom flask. The second portion of XAD-2 resin was transferred to a second 10-mL round-bottom flask. A volume of 5 mL of methanol was added to each flask followed by ultrasonication for 5 min. The solids were allowed to settle and the methanol was transferred by pipet to respective 25-mL round-bottom flasks. The extraction process using another 5-mL portion of methanol was repeated for each sample. The pooled methanol fractions for each sample were evaporated to dryness via rotary evaporation and the residues were reconstituted in 12 mL of hexane for analysis by GC/NPD.

5.2 Water

Water was extracted for fenoxycarb by passing 1 kg of water sample through a glass-microfiber filter into a 1-L dropping funnel. A chromatography column containing 5 g of XAD resin supported by 5 g of sea sand was successively conditioned with 80 mL of methanol and then 80 mL of acetone. Drying was accomplished by passing dry nitrogen through the column. The filtered water sample was passed through the XAD resin at a rate of 10 mL min^{-1}. After passage of the water sample through the resin, the remaining water was forced out of the column using dry nitrogen. The analyte was eluted from the column using 100 mL of ethyl acetate at 1 mL min^{-1} and collected in a 250-mL flask. The eluate was reduced to dryness using a rotary evaporator and reconstituted in 2 mL of ethyl acetate. Another column was prepared by adding 10 mL of hexane–ethyl acetate (23 : 2, v/v) in a chromatography column plugged with glass-wool. Next, 10 g of Florisil in a solvent slurry mixture were slowly added to the column by gently tapping the sides and the solvent level was allowed to drain to the top of the Florisil. A 2-mL fraction from the previous column step was quantitatively transferred to the Florisil column and the column was washed with 100 mL of ethyl acetate. The analyte was eluted using 100 mL of hexane–ethyl acetate (17 : 3, v/v) and collected in a 250-mL flask. This fraction was reduced to dryness and the residue was reconstituted using 0.5 mL of mobile phase for analysis using LC/UV.

Pond water was analyzed for fenoxycarb by partitioning 1 L of filtered (Whatman No. 2 filter) water sample with 75 mL of hexane. The partitioning step was repeated

twice and the pooled hexane fraction was dried through a bed of anhydrous sodium sulfate. The dried hexane was reduced to about 1 mL using rotary evaporation and quantitatively transferred to a concentration tube. This fraction was again reduced to about 1 mL and the sides of the concentration tube were rinsed with 2 mL of methanol. This fraction was then reduced to dryness and reconstituted in an appropriate volume of mobile phase for analysis using three-column switching LC/UV.

5.3 Soil

Soil was extracted for fenoxycarb by placing 20 g of sample in a 250-mL extraction jar with a Teflon-lined lid containing 20 mL of 1% phosphoric acid. The jar was allowed to stand for 20 min before adding 200 mL of acetone followed by mechanical shaking for 30 min. The extract was filtered through glass-fiber filters into a 500-mL side-arm filtering flask using 2×15 mL of acetone to rinse the extraction jar. This fraction was quantitatively transferred to a 500-mL evaporation flask and reduced in volume to 20–25 mL at a water-bath temperature of $<35\,^{\circ}C$ to remove all traces of acetone. Acetonitrile (30 mL) was added to the flask and swirled to mix before transfer to a 250-mL separatory funnel. A volume of 2×10 mL of acetonitrile–water (3 : 2, v/v) was used to rinse the evaporation flask. The acetonitrile–water mixture was partitioned with 50 mL of hexane (1 min). The hexane phase was separated from the aqueous phase and partitioned twice (for 1 min each time) with 10 mL of acetonitrile–water (3 : 2, v/v). The two acetonitrile–water solvent extracts were returned to the original acetonitrile–water extract and the hexane was discarded. The acetonitrile–water fraction was reduced to about 40 mL via rotary evaporation at a water-bath temperature of $<35\,^{\circ}C$ to remove the acetonitrile. To the remaining aqueous fraction was added 2 mL of concentrated ammonia solution and 20 mL of deionized water followed by transfer to a 125-mL separatory funnel. A volume of 50 mL of hexane was added to the evaporation flask for rinsing purposes before transfer to the separatory funnel. After phase separation, the hexane phase was dried through a bed of anhydrous sodium sulfate and collected in a clean 250-mL evaporation flask. The aqueous fraction was partitioned twice more, each time with 50 mL of hexane. The pooled and dried hexane fraction was reduced to dryness using rotary evaporation prior to reconstitution in an appropriate volume of hexane for GC analysis.

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5.4 Plant material

5.4.1 Pasture grass hay, forage, and cucurbits

Fenoxycarb was extracted from pasture grass hay, forage, and cucurbits (cucumbers, squash, and cantalope) by weighing a 25-g representative sample into a 16-oz wide-mouthed jar followed by the addition of 20 mL of 1% phosphoric acid. After waiting for 20 min, 200 mL of acetone were added. After waiting for 1 min, the sample was homogenized using a Polytron at a rate of 17 000–20 000 rpm for 2 min. The sample was then filtered using a 500-mL side-arm flask equipped with a Buchner funnel containing a glass-microfiber filter. The sample bottle was rinsed with 50 mL of acetone and the solvent was filtered. The extract was transferred to a 1-L round-bottom

flask and the acetone volume was reduced to about 18 mL using rotary evaporation at a water-bath temperature of <35 °C. The volume of the remaining aqueous fraction was measured and enough acetonitrile was added to obtain an acetonitrile : water ratio of 3 : 2 (v/v). This fraction was transferred to a 250-mL separatory funnel using 2 × 10 mL of acetonitrile–water (3 : 2, v/v) solvent mixture for rinsing purposes. The 1-L round-bottom flask was rinsed with 50 mL of hexane and the rinsate was added to the separatory funnel, which was then shaken for 1 min. The hexane fraction was removed and back-partitioned with 2 × 10 mL of acetonitrile–water (3 : 2, v/v) and these two portions were returned to the original aqueous acetonitrile fraction. The hexane layer was discarded. Acetonitrile was removed via rotary evaporation until the first drops of water were observed in the condenser (the aqueous volume should be similar to that previously, about 18 mL). Concentrated ammonia solution (2 mL) and water (20 mL) were added and the mixture was transferred to a 125-mL separatory funnel. The flask was rinsed with 50 mL of hexane and the rinsings were also added to the separatory funnel. After shaking for 1 min, the hexane phase was removed and transferred to a 250-mL round-bottom flask. The aqueous portion was partitioned twice more with 50 mL of hexane. The aqueous phase was discarded and the pooled hexane fraction was reduced to dryness using rotary evaporation. This fraction was reconstituted in 2 mL of hexane and analyzed by LV/UV for crop samples. Pasture grass and forage samples were subjected to further purification by preconditioning a silica gel SPE cartridge with 2–3 mL of hexane. The sample was loaded on to the SPE cartridge and the 250-mL round-bottom flask was rinsed with 2 × 2-mL portions of hexane, the rinsings also being added to the cartridge. The column was first eluted with 7 mL of dichloromethane–hexane–tetrahydrofuran (49 : 50 : 1, v/v/v) solvent mixture, which was discarded after passage through the cartridge. The analyte was eluted using an appropriate volume of the same elution solvent (typically 15–25 mL) and collected in a 50-mL round-bottom flask. Note that the fenoxycarb elution volume on each lot of silica gel SPE cartridges was profiled due to lot-to-lot variability. The eluate was reduced to dryness followed by reconstitution in 2 mL of hexane. A 5-mm deep layer of sodium sulfate was added to the top of a Florisil PrepSep cartridge and was then preconditioned using 2–3 mL of hexane–ethyl acetate (23 : 2, v/v). The 2-mL hexane fraction was loaded on to the Florisil PrepSep cartridge using 2 × 2-mL portions of hexane for rinsing purposes. The analyte was eluted with 50 mL of hexane–ethyl acetate (23 : 2, v/v) and collected in a 100-mL round-bottom flask. This fraction was reduced to dryness and reconstituted in an appropriate volume of mobile phase for LC analysis or in an appropriate volume of acetonitrile for GC/MS analysis.

5.4.2 Citrus, pome fruit, tree nut, fruiting vegetables, and cotton substrates

A 10-g representative sample (5-g sample for citrus oil or cotton substrates) was extracted by adding 150 mL of acetonitrile–water (4 : 1, v/v) to the sample in an 8-oz bottle and homogenized with a Polytron at high speed for 2 min. The extract was filtered through a Whatman No. 1 filter-paper into a 500-mL side-arm flask. The extraction bottle was rinsed with 50 mL of acetonitrile–water (4 : 1, v/v) for citrus and cottonseed oil (for molasses use 10 mL of water followed by 40 mL of acetonitrile for rinsing). The extract was transferred to a 500-mL separatory funnel and partitioned twice, each time with 50 mL of hexane for 1 min. The hexane fractions

were discarded. The extract was transferred to a 500-mL boiling flask and the acetonitrile was removed using rotary evaporation at a water-bath temperature of 35–40 °C (removal of all the acetonitrile is critical). For tree nut samples only, a Waters C_{18} Sep-Pak cartridge was conditioned with 10 mL of acetonitrile–water (3 : 2, v/v) and 10 mL of water at a flow rate of 1–3 drops per second (avoiding column dryness). The sample was loaded followed by washing with 25 mL of acetonitrile–water (1 : 4, v/v) and then 10 mL of acetonitrile–water (3 : 2, v/v). The analyte was eluted using 40 mL of acetonitrile–water (3 : 2, v/v). For all other samples (excluding tree nuts), the remaining aqueous portion after removing the acetonitrile was transferred to a 250-mL separatory funnel to which 50 mL of 0.5% sodium carbonate and 25 mL of water saturated with sodium chloride were added. For tree nuts, the eluate from the Waters Sep-Pak cleanup step was transferred to a 250-mL separatory funnel, and 30 mL of 0.5% sodium carbonate and 10 mL of water saturated with sodium chloride were added. All the samples were partitioned twice, each time with 50 mL of hexane. The pooled hexane fraction was dried through anhydrous sodium sulfate. The remaining aqueous portion was discarded. For tree nuts only, the dried hexane fraction was reduced to dryness and reconstituted in 2 mL of hexane for Florisil purification. The dried hexane fraction for all other samples was reduced to 5–15 mL using rotary evaporation for silica gel purification. A silica gel SPE cartridge was conditioned with 6 mL of ethyl acetate–hexane (1 : 4, v/v) and 6 mL of hexane at a flow rate of 1–2 drops per second. The hexane fraction was loaded on the column followed by addition of 6 mL of ethyl acetate–hexane (1 : 4, v/v) for washing purposes. The analyte was eluted using 12 mL of ethyl acetate–hexane (1 : 4, v/v) and collected in a 50-mL concentration tube. This fraction was reduced to dryness and reconstituted in 2 mL of hexane for Florisil cleanup, except for cucurbit and fruiting vegetable samples, which were reconstituted in acetonitrile–0.05 M potassium dihydrogenphosphate (1 : 1, v/v) for LC analysis. All other samples (including tree nuts) were further purified by conditioning a Florisil SPE cartridge with 3–5 mL of ethyl acetate–hexane (2 : 23, v/v) at a rate of 1–2 drops per second (avoiding column dryness). The hexane fraction was loaded on to the column and the concentration tube was rinsed in 2 × 2 mL of hexane, the rinsings being added to the column. The analyte was eluted with 50 mL of ethyl acetate–hexane (2 : 23, v/v) and collected in a 125-mL boiling flask. This fraction was reduced to dryness and reconstituted in 1 mL of acetonitrile–0.05 M potassium dihydrogenphosphate (1 : 1, v/v) for LC analysis.

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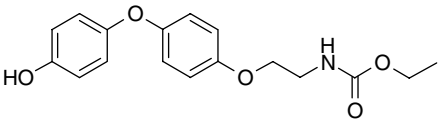
5.4.3 Oranges, onions, grapes, and tomatoes

A 0.5-g portion of sample was weighed into a mortar and gently blended with 0.5 g of silica-based sorbent containing C_8 or C_{18} functional groups (45–55-μm particle diameter range) to obtain a homogeneous mixture. The mixture was introduced into a 100 × 9-mm i.d. glass column. A 10-mL volume of dichloromethane–acetonitrile (3 : 2, v/v) was added to the column and allowed to elute dropwise under slight vacuum into a 15-mL conical-shaped graduated cylinder. This fraction was reduced to 0.5 mL using a gentle stream of nitrogen prior to analysis using LC/MS [with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI)]. This method was applicable to the analysis of 13 carbamate residues, including fenoxycarb.

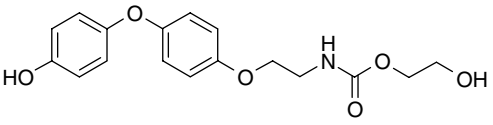
5.5 Animal material

5.5.1 Meat, milk, blood, and eggs

In addition to the parent fenoxycarb, residue methods for the two major metabolic products Ro-16-8797 {CGA-294850, ethyl *N*-2-[4-(4-hydroxyphenoxy) phenoxyethyl]carbamate, MW = 317.3} and Ro-17-3192 {CGA-294851, (2-hydroxyethyl)-*N*-2-[4-(4-hydroxyphenoxy)phenoxyethyl]carbamate, MW = 333.3} in animal by-products were also developed.



Ro-16-8797



Ro-17-3192

Fat, muscle, and blood samples (or liver, kidney, well-mixed milk, and egg homogenate) were extracted for fenoxycarb and its hydroxylated metabolites Ro-16-8797 and Ro-17-3192 by homogenizing 10 g of tissue or whole blood in 200 mL of acetonitrile [or 200 mL of water–acetonitrile (1 : 4, v/v)] for 2 min using a Polytron at high speed. The extract was then filtered through Whatman No. 1 filter-paper into a 500-mL side-arm flask. A volume of 50 mL of acetonitrile [or 50 mL of water–acetonitrile (1 : 4, v/v)] was used to rinse the extraction bottle and filter pad. This fraction was transferred to a 500-mL separatory funnel and partitioned twice using 50 mL of hexane each time. The hexane was discarded. For fat, muscle, and blood, the acetonitrile was removed using rotary evaporation at a water-bath temperature of 35–40 °C. For liver, kidney, milk, and eggs, 25 mL of water saturated with sodium chloride and 50 mL of toluene were added to the remaining portion of water–acetonitrile (1 : 4, v/v). The mixture was shaken for 1 min. The aqueous phase was discarded and the toluene phase was transferred to a 500-mL flat-bottom flask. This fraction was reduced to dryness. For all samples, 25 mL of 0.5% sodium carbonate were added and this fraction was swirled and sonicated to ensure complete dissolution and mixing prior to transfer to a 125-mL separatory funnel. A second 25-mL portion of 0.5% sodium carbonate was used for rinsing purposes. A volume of 2×25 mL of ethyl acetate–hexane (1 : 1, v/v) was used for rinsing purposes and also as partitioning solvent by shaking the separatory funnel for 1 min. After phase separation, the organic solvent was dried through a bed of prerinsed [using 25 mL of ethyl acetate–hexane (1 : 1, v/v)] sodium sulfate and collected in a 250-mL flat-bottom boiling flask. The aqueous portion was partitioned a second time using 50 mL of ethyl acetate–hexane (1 : 1, v/v) and dried through the same bed of sodium sulfate. The pooled and dried ethyl acetate–hexane fraction was reduced just to dryness using rotary evaporation and a water-bath temperature of 35–40 °C. A silica gel SPE cartridge was conditioned using 15 mL of ethyl acetate and 10 mL of dichloromethane (avoiding column drying) at a rate of 1–2 drops per second via gravity (a vacuum system was not used). To the residue contained in the flat-bottom flask were added 5 mL of dichloromethane, which was shaken vigorously and sonicated to ensure complete dissolution and mixing. This fraction was loaded on to the preconditioned silica column. A second 5-mL portion of dichloromethane was added to rinse the flask and also added to the silica gel SPE

cartridge. First, 10 mL of hexane and then 6 mL of ethyl acetate–hexane (1 : 4, v/v) were added to the flask, followed by transfer to the silica column. These wash solvents were discarded. Fenoxycarb and Ro-16-8797 were eluted with 8 mL of ethyl acetate–hexane (1 : 1, v/v) and collected in a 50-mL concentration tube. Ethyl acetate (15 mL) was then added to elute Ro-17-3192 and collected in a separate 50-mL concentration tube. The two fractions were reduced to dryness separately and each was reconstituted in acetonitrile–0.02 M potassium dihydrogenphosphate (1 : 1, v/v) for analysis using column switching LC.

6 Instrumentation

The following instrumental conditions have been shown to be suitable for the analysis of fenoxycarb. Other operating parameters may be employed provided that fenoxycarb is separated from sample interferences and the response is linear over the range of interest.

Operating conditions for air

Gas chromatograph	Hewlett-Packard 5890A Series II with HP-7673A autosampler and nitrogen–phosphorus detector.
Column	HP-17 fused silica, 10 m × 0.53-mm i.d., 2.0-μm film thickness
Temperatures	Injector 250 °C, detector 300 °C, oven 245 °C
Gas flow rates	Carrier gas, He, 11 mL min^{-1}; make-up gas, N_2, 22 mL min^{-1}; H_2, 2.7 mL min^{-1}; air, 108 mL min^{-1}
Volume injected	5 μL
Retention time for fenoxycarb	3.4 min

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Operating conditions for water

High-performance liquid chromatograph	Kontron 640 Kontron Uvikon 735 LC/UV detector
Column	125 × 4.6-mm i.d., SS, Nucleosil C_{18}, 5-μm particle size
Mobile phase	Isocratic, acetonitrile–0.05 M potassium dihydrogenphosphate (1 : 1, v/v, pH 4.5)
Detector wavelength	228 nm
Flow rate	1 mL min^{-1}
Retention time for fenoxycarb	10 min

Three-column switching

High-performance liquid chromatograph (pump 1)	Perkin-Elmer Series 410
Injector	Perkin-Elmer ISS-100 autosampler
Pumps 2 and 3	Waters M6000A
Switching valves	Valco Instruments, Model EL6W six-port with electronic actuator
Detector	Perkin-Elmer LS 40 fluorescence detector

Column 1	Phase-Sep S5 C1, 10 cm × 4.6-mm i.d., 5-μm particle size
Column 2	Phase-Sep S3 ODS-2, 10 cm × 4.6-mm i.d., 3-μm particle size
Column 3	Hamilton PRP-1, 15 cm × 4.1-mm i.d., 10-μm particle size
Mobile phase 1	Acetonitrile–0.05 M phosphate buffer (2 : 3, v/v) at 1 mL min^{-1}
Mobile phase 2	Acetonitrile–0.05 M phosphate buffer (1 : 1, v/v) at 1 mL min^{-1}
Mobile phase 3	Acetonitrile–0.05 M phosphate buffer (13 : 7, v/v) at 1 mL min^{-1}
Injection volume	200 μL
Excitation/emission wavelengths	230/300 nm
Retention time for fenoxycarb	Column 1, 8.9 min; column 2, 17.3 min; column 3, 23.6 min

Operating conditions for soil

Gas chromatograph	Varian 3400 with thermionic specific detector
Column	Restek Rtx-5, 30-m × 0.53-mm i.d., 1.5-μm film thickness
Temperatures	Injector: initial 50 °C (held 0 min), increased at 200 °C min^{-1} to 250 °C (held 17.5 min) Detector: 300 °C Oven: initial 50 °C, increased at 25 °C min^{-1} to 250 °C
Gas flow rates	Air, 180 mL min^{-1}; He, 30 mL min^{-1}; H_2, 4.5 mL min^{-1}
Volume injected	4–5 μL
Retention time for fenoxycarb	1.25 min

Operating parameters for pasture grass hay, forage, cucumbers, squash, and cantalope

High-performance liquid chromatograph	Perkin-Elmer Model ISS-200 automatic HPLC sampler Kratos Spectroflow 400 LC pumps ABI Model 783 variable-wavelength ultraviolet (UV) detector Valco Instruments, six-port switching valve
Column 1	Spherisorb C1, 100 × 4.6-mm i.d., 5-μm particle size (Fisher, Cat. No. 05-692-547)
Column 2	Spherisorb ODS2, 150 × 4.6-mm i.d., 3-μm particle size (Fisher, Cat. No. 05-692-536)
Mobile phase 1	Acetonitrile–0.05 M potassium dihydrogenphosphate (2 : 3, v/v), 1 mL min^{-1}
Mobile phase 2	Acetonitrile–0.05 M potassium dihydrogenphosphate (2 : 3, v/v), 1 mL min^{-1}

Detector wavelength	228 nm
Injection volume	40 μL
Retention time for fenoxycarb	Column 1, 7 min; column 2, 12 min
Gas chromatograph/ mass spectrometer	Hewlett-Packard 6890 Series gas chromatograph with Model 5973 mass-selective detector
Column	DB-1701, 30 m × 0.25-mm i.d., 0.15-mm film thickness (J&W Scientific, Cat. No. 1220731)
Temperatures	Injector : 200 °C Detector : 280 °C Oven : initial 50 °C (held 1 min), ramp A 50 °C min^{-1} to 250 °C (held 0 min), ramp B 10 °C min^{-1} to 300 °C (held 5 min)
Gas flows rates	Pressure 10 psi, EPP mode, column (He) 1.2 mL min^{-1}; purge 60 mL min^{-1}, purge time 0.6 min
Volume injected	2 μL
Retention time for fenoxycarb	1.25 min
Selected ion monitoring	Target ion m/z 116, qualifier ions m/z 186 and 301

Citrus, pome fruit, tree nuts, fruiting vegetables, and cotton substrates

High-performance liquid chromatograph	Two Waters Model 501 pumps Perkin-Elmer Model ISS-100 automatic sampler Kratos ABI Spectroflow Model 783 ultraviolet/visible (UV/VIS) detector
Column 1	Phase Separation Spherisorb C1, 100 × 4.6-mm i.d., S5
Column 2	Phase Separation Spherisorb ODS2, 150 × 4.6-mm i.d., S3
Mobile phase 1	Acetonitrile–0.05 M potassium dihydrogenphosphate (2 : 3, v/v)
Mobile phase 2	Acetonitrile–0.05 M potassium dihydrogenphosphate (1 : 1, v/v) For tree nuts: acetonitrile–methanol–0.05 M potassium dihydrogenphosphate (8 : 5 : 7, v/v/v)
Detector wavelength	225 nm
Flow rate	1 mL min^{-1}
Injection volume	50 μL
Retention time of fenoxycarb	About 13 min for tree nut substrates and 20 min for all other substrates

Operating parameters for oranges, onions, grapes, and tomatoes

High-performance liquid chromatograph	Hewlett-Packard 1100 Series LC-MSD equipped with an atmospheric pressure ionization (API) source (APcI or ESI)
Column	Spherisorb C_8, SS, 150 × 4.6-mm i.d., 3-μm particle size (and a LiChrosorb RP-8 guard column)

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Mobile phase (APCI)	Methano–water (1 : 1, v/v), isocratic for 5 min, linear to 60% methanol for 5 min, held for 5 min, then to 90% methanol in 5 min, held for 7 min, 1 mL min^{-1} (other conditions may be more appropriate if analyzing solely for fenoxycarb)
Mobile phase (ESI)	Methanol–water, (1 : 1, v/v), isocratic for 15 min, to 70% methanol in 5 min, held for 5 min, then to 90% methanol in 5 min, held for 5 min
APcI (+ mode)	302 $[M + H]^+$, 230 $[M + H - (CH_3)_2NCO]^+$ Vaporizer temperature, 325 °C; nebulizer gas, N_2, at 4.1 bar; drying gas, N_2, at 4 L min^{-1} and temperature 350 °C; capillary voltage, 4000 V; corona current, 4 μA
ESI (+ mode)	302 $[M + H]^+$ Gas temperature, 350 °C at 13 L min^{-1}; nebulizer gas pressure, 30 psi; capillary voltage, 4000 V
Injection volume	5 μL
Retention time for fenoxycarb	26–30 min

Operating parameters for meat, milk, blood, and eggs

High-performance liquid chromatograph	Two Waters Model 501 pumps Perkin-Elmer Model ISS-100 automatic sampler Kratos ABI Spectroflow Model 783 UV/VIS detector, VICI EQ60 LC switching valve
Column 1	Supelcosil LC-CN, 33 × 4.6-mm i.d., 5-μm particle size
Column 2	Supelcosil LC-1, 250 × 4.6-mm i.d., 5-μm particle size
Mobile phase 1	0.02 M potassium dihydrogenphosphate (adjusted to pH 3 with phosphorous acid)–methanol (7 : 3, v/v)
Mobile phase 2	0.02 M potassium dihydrogenphosphate (adjusted to pH 3 with phosphorous acid)–methanol–acetonitrile (12 : 5 : 3, v/v/v)
Detector wavelength	235 nm
Flow rate	1.5 mL min^{-1}
Injection volume	50 μL
Retention time ranges	Fenoxycarb: 14–27 min Ro16-8797: 10–16 min Ro-17-3192: 5.6–8 min

7 Evaluation

7.1 Method

Quantification was performed in all cases using the external calibration method. A series of standards were injected and the responses plotted against their known concentrations. Peak responses in samples were compared with the calibration plots

to obtain the amount found (nanograms). A fresh calibration plot was generated with each analytical set of samples.

7.2 Recoveries, limit of detection (LOD) and limit of quantitation (LOQ)

The lower practical level of quantitation for fenoxycarb in air is 10 $\mu g\,m^{-3}$ using the described sampling rates and times. The average recovery obtained from fortifying and extracting the OVS tubes was 94%.

The recoveries of fenoxycarb (CGA-114597) from water ranged from 71 to 90% at fortification levels from 0.05 to 1 $\mu g\,L^{-1}$. The LOQ was 0.05 $\mu g\,L^{-1}$.

The average recovery obtained for fenoxycarb when the analysis of pond water was performed using three-column switching LC/fluorescence detection was 100% at fortification levels ranging from 0.001 to 10 $\mu g\,L^{-1}$. The LOQ and LOD were 0.001 $\mu g\,L^{-1}$ and 0.4 ng injected, respectively.

The average recoveries for fenoxycarb in soil were 89, 105, and 104% for soil collected in California, Washington, and Georgia, respectively, at fortification levels ranging from 0.01 mg kg^{-1} to 1.0 mg kg^{-1}. The LOQ and LOD were 0.01 mg kg^{-1} and 0.2 ng injected, respectively.

The average recovery obtained for fenoxycarb in pasture grass at the method LOQ was 82%. At all fortification levels, the average recovery for pasture grass was 81%. For cucurbits (cucumbers, squash, cantalope) the average recovery at the method LOQ were 102% and for all fortification levels is 99%. The LOQ and LOD were 0.01 mg kg^{-1} and 2 ng injected, respectively, using LC. The average recovery for the analysis of pasture grass forage and pasture grass hay using GC/MS were 97% and 85%, respectively. The recovery data obtained using LC/UV and GC/MS were comparable. However, confirmatory evidence was obtained using GC/MS.

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The average recoveries and standard deviations for the many citrus, pome fruit, tree nut, fruiting vegetables, and cotton substrate sample types were acceptable when fortified at concentration levels ranging from 0.01 to 4 mg kg^{-1}. The LOQ of the method was 0.01 mg kg^{-1}, except for citrus oil (0.02 mg kg^{-1}), and the LOD was 1.25 ng injected.

The fenoxycarb recoveries for orange, onion, grape, and tomato samples ranged from 63 to 70%. The LOQ and LOD were 0.01 mg kg^{-1} and 0.005 mg kg^{-1}, respectively, when using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI/MS).

Average recoveries for fenoxycarb, Ro-16-8797, and Ro-17-3192 for all animal sample substrates ranged from 80% (beef kidney) to 111% (goat kidney), 76% (goat milk) to 93% (beef omental fat), and 56% (dairy milk) to 76% (beef perirenal fat), respectively. The LOQ and LOD were 0.01 $\mu g\,g^{-1}$ and 2.5 ng injected, respectively.

7.3 Calculation of residues

Water

$$\mu g\,kg^{-1} = AC100/BDR$$

where A = ng found (from the calibration plot), B = injection volume, C = final fraction volume, D = weight of sample extracted, and R = percentage recovery (expressed as a decimal).

Pond water

$$\text{ppb} = \text{ng found/mL injected}$$

where ng found is taken from the calibration plot,

$$\text{g injected} = g\,V_\text{i}/\,V_\text{f}$$

where g = weight of sample (1.0 mL = 1.0 g), V_i = volume of sample injected into the LC system, and V_f = final fraction volume.

Soil

$$\text{ppm } (\mu\text{g g}^{-1}) = (\mu\text{g g}^{-1} \text{ equivalents from calibration plot})\,(V_\text{f}/\,W_\text{s})\,(\text{dilution factor})$$

where V_f = final fraction volume (mL) and W_s = sample weight (g).

Plant and animal sample substrates

$$\text{mg injected} = G\,V_\text{a}V_\text{i}/[(V_\text{e} + W(M/100)]V_\text{f}$$

where G = mg sample extracted, V_a = aliquot volume, V_e = extraction volume, V_i = injection volume, M = sample moisture (%), and W = sample weight (g).

$$\text{ppm} = \text{ng found from calibration plot/mg sample injected}$$

Reference

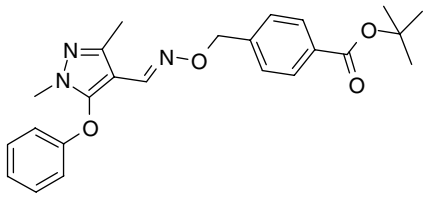
1. M. Fernandez, Y. Pico, and J. Manes, *J. Chromatogr. A,* **871**, 43 (2000).

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Fenpyroximate

Materials to be analyzed	Plants (citrus, apple, grape, tea, hop, soybean, cucumber, eggplant, ornamentals), soil and water
Instrumentation	Gas-chromatographic determination [nitrogen–phosphorus detection (NPD)] for plant materials, soil and water

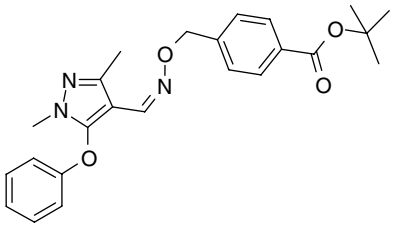
1 Introduction

Chemical name (IUPAC)	*tert*-Butyl (*E*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-yl-methyleneaminooxy)-*p*-toluate
Structural formula	
Empirical formula	$C_{24}H_{27}N_3O_4$
Molar mass	421.5
Melting point	99–102 °C
Vapor pressure	7.4×10^{-6} Pa at 25 °C
Solubility	Water 0.0146 mg L^{-1} at 20 °C Methanol 15 g L^{-1} at 25 °C Acetone 150 g L^{-1} at 25 °C Dichloromethane 1307 g L^{-1} at 25 °C Chloroform 1197 g L^{-1} at 25 °C Tetrahydrofuran 737 g L^{-1} at 25 °C
Stability	Stable in acidic and alkaline conditions
Other properties	Log $K_{ow} = 5.01$
Use pattern	Fenpyroximate exhibits acaricidal and knockdown activities on phytophagous mites, such as *Tetranychus urticae* Koch (two-spotted spider mite) and *Panonychus citri*[1,2] in citrus, apple, pear, peach, grape, etc. Fenpyroximate inhibits the mitochondrial NADH-Co Q reductase, which induces a decrease in ATP content and morphological changes in mitochondria and ultimately shows the acaricidal and knockdown activities.[3]

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Handbook of Residue Analytical Methods for Agrochemicals.
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Regulatory position The major metabolite of fenpyroximate in plants, soil and water is its *Z*-isomer (M-1). The target analytes are considered to be fenpyroximate and M-1 in plant, soil and water samples.



2 Outline of method

Fenpyroximate and M-1 residues in the plant (apple, grape, etc.) and soil samples can be analyzed using the multi-residue method 'Method DFG S19'[4] with some minor deviations. In this method, gel permeation chromatography (GPC) is effectively used as the cleanup procedure. Residues in the water sample can be analyzed by a simpler method.

Two different types of residue analytical methods of fenpyroximate and its metabolite (M-1) are provided for plant (apple, grape, etc.), soil and water:

A *'Multi-residue analytical method (for plant and soil)'* is provided for plants (apple, grape etc.) and soil samples. After GPC cleanup, fenpyroximate and M-1 are analyzed simultaneously using gas chromatography (GC)/NPD.
B *'GC/NPD method (for water)'* is provided for water samples to determine fenpyroximate and M-1 simultaneously using GC/NPD.

3 Multi-residue analytical method (for plants and soil)

3.1 Apparatus

Erlenmeyer flask, 500-mL
Buchner funnel
Round-bottom flask, 500-mL
Separatory funnel, 500-mL
Rotary evaporator
Solid-phase extraction (SPE) cartridge, LC Si 2-g, 12-mL (Supelco), or equivalent
ABC Laboratories Model SP 1000 gel permeation chromatograph system equipped with a 2.5 × 32.0 cm glass column of Bio-Beads S-X3 Select 200–400 mesh (ca 50 g, Bio-Rad Laboratories) preconditioned with ethyl acetate-cyclohexane (1 : 1, v/v), or equivalent
Fused-silica capillary column, DB-1, 15 m × 0.32-mm i.d., 0.25-μm film thickness, 100% dimethylpolysiloxane (*for plant materials*)

Fused-silica megabore column, DB-1, 15 m × 0.53-mm i.d., 0.15-μm film thickness, 100% dimethylpolysiloxane (*for soil*)
Varian Model 3400 gas chromatograph with capillary split/splitless inlet with nitrogen–phosphorus detector equipped with a Model 8200 autosampler, or equivalent

3.2 Reagents and supplies

Filter aid, e.g., Celite 545 (Roth)
Acetone, residue analysis grade
Dichloromethane, residue analysis grade
Ethyl acetate, residue analysis grade
Cyclohexane, residue analysis grade
n-Hexane, residue analysis grade
Toluene, residue analysis grade
Sodium sulfate, anhydrous, reagent grade

3.3 Procedure

3.3.1 Extraction

For plant materials. Weigh 50 g of the plant sample into a 500-mL Erlenmeyer flask, add 10 g of Celite and extract with 60 mL of the distilled water and 200 mL of acetone (the calculated acetone : water ratio remains constant at 2 : 1, v/v). Filter the extract by suction through a Buchner funnel.

For soil. Extract 50 g (dry weight, containing about 5 g of the water) of the soil sample similarly as described above, using 95 mL of the distilled water.

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3.3.2 Cleanup

Partitions

Transfer an aliquot of the extract (around 250 mL) into a 500-mL separatory funnel, add 20 g of the sodium chloride and shake vigorously. Extract the mixture with 100 mL of dichloromethane. Dry the extracts with anhydrous sodium sulfate, filter through a cotton-wool plug and measure the volume. Transfer an aliquot of the extract (around 260 mL) into a 500-mL round-bottom flask and concentrate to around 5 mL with a rotary evaporator. Transfer the sample to a centrifuge tube with 5 mL of ethyl acetate and partition. Repeat the partitioning twice more, combine the ethyl acetate fractions and concentrate to dryness with a rotary evaporator.

Gel permeation chromatography (GPC)

Dissolve the residue obtained above with 4.0 mL of ethyl acetate and then add 4.0 mL of cyclohexane. Apply 5 mL of this sample extract to the GPC system, elute with

ethyl acetate–cyclohexane (1 : 1, v/v) at a flow rate of 5.0 mL min^{-1} and collect the elution volume fraction 100–135 mL. Evaporate this fraction to dryness.

Chromatography on a silica gel cartridge

Condition a 2-g/12-mL silica gel SPE cartridge (layered with ca 2 cm of anhydrous sodium sulfate) with n-hexane prior to use. Reconstitute the residue from the GPC with 5.0 mL of dichloromethane–n-hexane (1 : 1, v/v) and apply to an SPE cartridge. Wash the cartridge with 1 mL of n-hexane followed by 6 mL of n-hexane–toluene (13 : 7, v/v), 6 mL of toluene and then 6 mL of toluene–acetone (19 : 1, v/v). Elute the sample from the cartridge with 6 mL of toluene–acetone (4 : 1, v/v). Concentrate the eluate and reconstitute the sample with 1.0 mL of toluene for GC/NPD analysis.

3.3.3 Determination

Inject an aliquot of the GC-ready sample solution into the GC/NPD system.

Operating conditions for GC/NPD (*plant materials*)

Gas chromatograph	Model 3400, Varian
Sample injector	Splitless mode
Column	Fused-silica capillary column, DB-1, 15 m × 0.32-mm i.d., 0.25-μm film thickness
Column temperature	Initial 200 °C, held for 2 min, increased at 5 °C min^{-1} to 250 °C and then at 30 °C min^{-1} to 350 °C, held for 3 min
Injection port temperature	240 °C
Detector temperature	280 °C
Detector	Nitrogen–phosphorus detector
Gas flow rate	Helium carrier gas, 25 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 175 mL min^{-1}
Injection volume	2 μL

Operating conditions for GC/NPD (*soil*)

Gas chromatograph	Model 3700, Varian
Sample injector	Splitless mode
Column	Fused-silica megabore column, DB-1, 15 m × 0.53-mm i.d., 0.15-μm film thickness
Column temperature	Initial 150 °C, held for 2 min, increased at 10 °C min^{-1} to 240 °C, held for 5 min
Injection port temperature	230 °C
Detector temperature	250 °C
Detector	Nitrogen–phosphorus detector
Gas flow rates	Helium carrier gas, 30 mL min^{-1} Hydrogen, 4.5 mL min^{-1} Air, 175 mL min^{-1}
Injection volume	5 μL

3.4 Evaluation

3.4.1 Method

Standardization

For plant materials. Peaks of M-1 and fenpyroximate usually appear at retention times around 7.4 and 8.2 min, respectively. Plot the peak areas against the concentrations of the analytes.

For soil. Peaks of M-1 and fenpyroximate usually appear at retention times around 9.0 and 9.5 min, respectively. Plot the peak heights against the concentrations of the analytes.

Detection of sample residues

Inject the cleaned-up sample into the GC/NPD system operated under the same conditions as employed for standardization. Compare the peak areas or heights of the analytical samples with the calibration curve. Determine the concentrations of M-1 and fenpyroximate present in the sample.

3.4.2 Recoveries, limit of detection and limit of determination

For plant materials. Recoveries of M-1 and fenpyroximate are evaluated as combined recoveries of these two compounds because of the chemical conversion potential of M-1 to fenpyroximate.

With fortification levels between 0.01 and 0.50 mg kg^{-1}, average recoveries of M-1 and fenpyroximate from untreated apple and grape samples are 87 and 91% with the limits of determination being 0.01 and 0.05 mg kg^{-1}, respectively, for the apple and grape samples.

For soil. With fortification levels between 0.01 and 0.50 mg kg^{-1}, average recoveries of M-1 and fenpyroximate from untreated soil samples are 88 and 98%, respectively, with the limit of determination being 0.01 mg kg^{-1}.

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3.4.3 Calculation of residues

Calculate the concentrations of the analyte (M-1 and fenpyroximate) in plant materials or soil samples (mg kg^{-1}) with the following equation:

$$\text{Analyte concentration} = \frac{A \times \frac{8}{5} \times V}{W}$$

where

A = concentration of the analyte in the final solution (mg L^{-1})
V = volume of the final solution (mL)
W = weight of analysis sample (g)
8 = total volume of the GPC-ready sample (mL)
5 = volume of the sample applied to the GPC cleanup (mL)

4 Multi-residue analytical method (for water)

4.1 Apparatus

Separatory funnel, 1-L
Round-bottom flask, 250-mL
Rotary evaporator
Fused-silica megabore column, DB-1, 15 m × 0.53-mm i.d., 0.15-μm film thickness, 100% dimethylpolysiloxane
Hewlett-Packard 5890 gas chromatograph with capillary split/splitless inlet with nitrogen–phosphorus detector equipped with a Model 7673 autosampler

4.2 Reagents and supplies

Dichloromethane, reagent grade
Ethyl acetate, reagent grade
Sodium sulfate, anhydrous, reagent grade

4.3 Procedure

4.3.1 Extraction

Extract the sample of water (1000 mL) three times each with 50 mL of dichloromethane. Dry with anhydrous sodium sulfate and remove the combined organic phase by rotary evaporation. Dissolve the remaining residue with 1 mL of ethyl acetate for GC/NPD analysis.

4.3.2 Cleanup

Cleanup is not needed for water samples.

4.3.3 Determination

Inject an aliquot of the GC-ready sample solution into the GC/NPD system

Operating conditions for GC/NPD

Gas chromatograph	Model 5890, Hewlett-Packard
Sample injector	Splitless mode
Column	Fused-silica megabore column, DB-1, 15 m × 0.53-mm i.d., 1.5-μm film thickness
Column temperature	Initial 150 °C, held for 2 min, increased at 7 °C min^{-1} to 250 °C, held for 8 min
Injection port temperature	250 °C
Detector temperature	270 °C
Detector	Nitrogen–phosphorus detector

Gas flow rates	Helium, 30 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 120 mL min^{-1}
Injection volume	4 μL

4.4 Evaluation

4.4.1 Method

Standardization

Peaks of M-1 and fenpyroximate usually appear at retention times around 13.8 and 14.4 min, respectively. Plot the peak heights against the concentrations of the analytes.

Detection of sample residues

Inject the cleaned-up sample into the GC/NPD system operated under the same conditions as employed for standardization. Compare the peak heights of the analytical samples with the calibration curve. Determine the concentrations of M-1 and fenpyroximate present in the sample.

4.4.2 Recoveries, limit of detection and limit of determination

With fortification levels between 0.05 and 5.0 μg L^{-1}, average recoveries of M-1 and fenpyroximate from untreated water samples are 94 and 97%, respectively, with the limit of determination being 0.05 μg L^{-1}.

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4.4.3 Calculation of residues

Calculate the concentrations of the analyte (M-1 and fenpyroximate) in water samples (μg L^{-1}) with the following equation:

$$\text{Analyte concentration} = \frac{A \times V}{W}$$

where

A = concentration of the analyte in the final solution (μg L^{-1})
V = volume of the final solution (mL)
W = volume of analysis sample (mL)

References

1. H. Hamaguchi, T. Oshima, H. Takaishi, Y. Akita, T. Konno, and O. Kajihara, 'Synthesis and acaricidal activities of fenpyroximate (NNI-850) and its related compounds,' in "Abstract Papers of the 7th International Congress of Pesticide Chemistry," Vol. 1, p. 43 (1990).

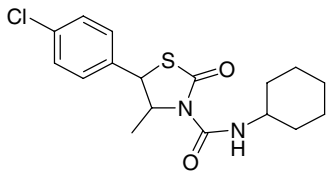
2. T. Konno, K. Kuriyama, H. Hamaguchi, and O. Kajihara, 'Fenpyroximate (NNI-850), a new acaricide,' in "Proceedings of the 1990 Brighton Crop Protection Conference," Vol. 2, pp. 71–78 (1990).
3. K. Motoba, T. Suzuki, and M. Uchida, *Pestic. Biochem. Physiol.,* **43**, 37 (1992).
4. W. Specht, 'Organochlorine, organophosphorus, nitrogen-containing and other pesticides, S 19,' in "Manual of Pesticide Residue Analysis (DFG, Deutsche Forschungsgemeinschaft, Pesticides Comm.)," ed. H.-P. Their and H. Zeumer, VCH, Weinheim, Vol. 1, pp. 383–400 (1987).

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Hexythiazox

Materials to be analyzed	Apple, pear, peach, citrus, grape, strawberry, cucumber, green pepper, eggplant and green tea
Instrumentation	High-performance liquid chromatographic determination for plant materials

1 Introduction

Chemical name (IUPAC)	(4*RS*,5*RS*)-5-(4-Chlorophenyl)-*N*-cyclohexyl-4-methyl-2-oxothiazolidine-3-carboxamide
Structural formula	
Empirical formula	$C_{17}H_{21}ClN_2O_2S$
Molar mass	352.6
Melting point	108–108.5 °C
Boiling point	Not applicable
Vapor pressure	3.38×10^{-6} Pa at 20 °C
Solubility	Water 0.5 mg L^{-1} at 25 °C. Readily soluble in organic solvents such as acetone, acetonitrile, chloroform and methanol
Stability	Stable in an acidic to neutral aqueous solution. Unstable in strongly basic conditions
Use pattern	Hexythiazox is a thiazolidinone acaricide which has ovicidal, larvicidal and nymphicidal activities against many kinds of phytophagous mites infesting pome fruits, stone fruits, citrus, vegetables and other food crops. On these mites, hexythiazox works by both contact and stomach action
Regulatory position	The residue definition of hexythiazox for plant/crops is for hexythiazox itself

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2 Outline of method

Plant materials are homogenized with methanol. Hexythiazox residue is extracted with hexane and then transferred to acetonitirile by liquid–liquid partitioning. The acetonitirile is removed by rotary evaporation and the sample is cleaned up using Florisil PR column chromatography. The concentrated eluate is subjected to high-performance liquid chromatography (HPLC) analysis.

3 Apparatus

High-speed blender fitted with leak-proof glass jar and explosion-proof motor
Balances
Macerator (Polytron)
Laboratory mechanical shaker
Separatory funnel, 500-mL
Filter paper
Erlenmeyer flask, 300-mL
Round-bottom flasks, 300-mL
Rotary vacuum evaporator, 40 °C bath temperature
Glass tube for column chromatography, 12-mm i.d., 300-mm length
High-performance liquid chromatograph

4 Reagents

Methanol, guaranteed reagent grade
Acetone, guaranteed reagent grade
Celite (No. 545)
Sodium chloride, guaranteed reagent grade
Hexane, guaranteed reagent grade
Sodium hydroxide, guaranteed reagent grade
Sodium sulfate, anhydrous, guaranteed reagent grade
Acetonitrile, guaranteed reagent grade
Florisil, nonactivated (Florisil PR)

5 Sampling and preparation

5.1 Green tea

Grind leaves with dry-ice using a high-speed blender.

5.2 Fruits and vegetables

Cut into pieces with a kitchen knife.

6 Procedure

6.1 Extraction

Homogenize 50 g of a prepared sample with 100 mL of methanol in a macerator for 3 min. In the case of green tea (powder), homogenize 10 g of a prepared sample with 100 mL of acetone–water (7 : 3, v/v) and shake for 30 min with a mechanical shaker.

Filter the homogenate through a Celite layer (1–2 cm thickness) under reduced pressure. Wash the vessel and cake with 50 mL of methanol [acetone–water (7 : 3, v/v) for green tea] and filter the washings through the same Celite layer. Combine these filtrates and transfer to a 500-mL separatory funnel. Add 100 mL of 5% sodium chloride solution to the filtrate and extract twice with 100 mL of hexane for 5 min using a shaker. Transfer the hexane to another 500-mL separatory funnel and wash twice with 100 mL of 0.01 M sodium hydroxide solution for 2 min using a shaker. Discard the alkaline phase. Filter the hexane phase through a filter paper with anhydrous sodium sulfate, collect the hexane in a 300-mL Erlenmeyer flask and transfer the hexane to a 500-mL separatory funnel. Extract twice with 100 mL of acetonitrile for 5 min using a shaker. Combine the acetonitrile extracts in a 300-mL round-bottom flask and remove the acetonitrile to near dryness in a water-bath at ca 40 °C by rotary evaporation. Dissolve the residue in 5 mL of hexane.

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6.2 Cleanup

Transfer the residue on to the top of the column packed with 10 g of 5% water containing Florisil PR with the aid of hexane. Rinse the column with 95 mL of hexane and then with 100 mL of acetone–hexane (1 : 99, v/v). Discard the column washings. Elute hexythiazox with another 150 mL of acetone–hexane (1 : 99, v/v) in a 300-mL round-bottom flask and concentrate the eluate to near dryness by rotary evaporation at 40 °C. Prepare the HPLC-ready sample solution by dissolving the plant material residue in acetonitrile.

6.3 Determination

High-performance liquid chromatograph	Model LC-3A, Shimadzu
Column	Stainless-steel column, 4.6-mm i.d., 250-mm length
Stationary phase	Zorbax ODS
Mobile phase	Acetonitrile–water (7 : 3, v/v)
Flow rate	1.0–1.5 mL min^{-1}
Column temperature	40 °C
Detection	UV detector (SPD-2A) at 225 nm
Injection volume	20 μL

7 Evaluation

7.1 Method

Quantification is performed by the calibration technique. Construct a new calibration curve with hexythiazox standard solutions using acetonitrile for each set of analyses. Inject 20-μL aliquots of the standard solution using acetonitrile. The hexythiazox peak usually appears at a retention time around 13 min. Plot the peak heights against the injected amount of hexythiazox.

7.2 Recoveries, limit of detection and limit of determination

With a fortification level of 0.05 mg kg^{-1} (0.125 mg kg^{-1} for green tea), recoveries from untreated plant matrices ranged from 83 to 96%. The limit of detection (LOD) is 0.01 mg kg^{-1} (0.05 mg kg^{-1} for green tea).

7.3 Calculation of residues

The amount of hexythiazox residue (R, mg kg^{-1}) in the sample is calculated by the following equation:

$$R = C \times V/G$$

where

C = concentration of hexythiazox in the final solution (μg mL^{-1})
V = final sample volume (mL)
G = original sample weight (g)

8 Important point

When biological samples are stored at −30 °C, hexythiazox residue in different crops is stable for more than 1 year.

Reference

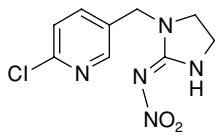
M. Tokieda, T. Tachibana, S. Kobayashi, T. Gomyo, and S. Ono, *J. Pestic. Sci.,* **12**, 711 (1987).

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Imidacloprid

Materials to be analyzed	Groundwater from wells and lysimeters
Instrumentation	Liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS)

1 Introduction

Chemical name (IUPAC)	1-(6-Chloro-3-pyridinyl)methyl-*N*-nitroimidazolidin-2-ylidenamine
Structural formula	
Empirical formula	$C_9H_{10}ClN_4O_2$
Molecular weight	255.7
Melting point	144.5 °C
Boiling point	Decomposes
Vapor pressure	1.50×10^{-9} Torr (20 °C)
Solubility (g L^{-1})	Water 0.51 g L^{-1} (ambient temperature). Solvents: dimethylformamide >200, dichloromethane 50–100, acetonitrile 50–100, acetone 20–50, isopropyl alcohol 1–2, toluene 0.5–1, hexane <0.1 g L^{-1}
Stability (hydrolysis half-time)	Stable at pH 5 and 7 (<1 yr), >250 h (pH 9)
Use pattern	Seed, foliar and soil application for sucking insects, some beetles, bollweevils and leafminers for fruits, vegetables, field crops, trees, ornamentals and turf. Used as a termaticide, flea control on domestic pets and ant bait.
Regulatory position	Regulated metabolites in groundwater include the parent, urea, guanidine and guanidine olefin metabolites. The drinking water health advisory level (HAL) is 399 μg L^{-1}.

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2 Outline of method

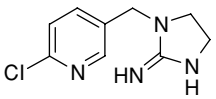
Water samples, received from the respective groundwater trials, are analyzed by direct aqueous injection (DAI) by LC/ESI-MS/MS. A 1-mL volume of the water is pipetted into a 1.8-mL autosampler vial. The internal standard solution is added (200 µL) and mixed. The vials are capped and analyzed by LC/ESI-MS/MS using the selected reaction monitoring (SRM) mode.

3 Reagents and standards

Water, high-performance liquid chromatography (HPLC)-grade high-purity solvent
Methanol (MeOH), HPLC-grade high-purity solvent
Acetonitrile (ACN), HPLC-grade high-purity solvent
Formic acid
Nitrogen, sublimed from liquid

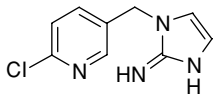
3.1 Native standards

Common name Guanidine



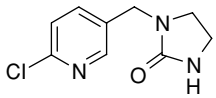
Molecular weight 210.7 (free base)
Chemical name 1-(6-Chloropyridin-3-ylmethyl)-2-iminoimidazolidine

Common name Olefinic guanidine



Molecular weight 208.7
Chemical name 1-[(6-Chloro-3-pyridinyl)methyl]-1*H*-imidazol-2-amine hydrochloride

Common name Urea



Molecular weight 211.7
Chemical name 1-[(6-Chloro-3-pyridinyl)methyl]-2-imidazolidinone

3.2 Internal standards

Common name $[D_3,{}^{13}C_1]$Imidacloprid
Molecular weight 259.7
Chemical name $[D_3,{}^{13}C_1]$-1-(6-Chloropyridin-3-ylmethyl)-*N*-nitro-2-iminoimidazolidine

Common name	$[D_3,^{13}C_1]$Guanidine
Molecular weight	214.7 (free base)
Chemical name	$[D_3,^{13}C_1]$-1-[(6-Chloro-3-pyridinyl)methyl]-4,5-dihydro-1*H*-imidazol-2-amine trifluoracetate
Common name	$[D_3,^{13}C_1]$Olefinic guanidine
Molecular weight	212.7
Chemical name	$[D_3,^{13}C_1]$-1-[(6-Chloro-3-pyridinyl)methyl]-1*H*-imidazol-2-amine trifluoroacetate
Common name	$[D_3,^{13}C_1]$Urea
Molecular weight	215.7
Chemical name	$[D_3,^{13}C_1]$-1-[(6-Chloro-3-pyridinyl)methyl]-2-imidazolidinone

3.3 Native stock solutions

Prepare stock solutions of each native analyte (nominally 0.2 mg mL^{-1}, 200 ppm) by dissolving reference material in aqueous methanol (1 : 1, v/v). The reference materials should be stored in a refrigerator and protected from light when not in use.

3.4 Mixed standard solution

From the stock solutions of the individual native analytes, prepare a mixed fortification solution at 50 μg mL^{-1} in methanol. Take an aliquot of this solution and dilute with aqueous methanol (1 : 1, v/v) to obtain a 0.25 μg mL^{-1} solution that will be used for fortifications.

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3.5 Internal standard stock solutions

Prepare stock solutions (nominally 0.1 mg mL^{-1}, 100 ppm) of each labeled analyte (to be used as internal standards), by dissolving reference material in methanol. The reference materials should be stored in a refrigerator and protected from light when not in use.

3.6 Mixed internal standard solution

From the stock solutions of the individual labeled analytes, prepare a mixed fortification solution at 50 μg mL^{-1} in methanol. Take an aliquot of this solution and dilute to 0.5 μg mL^{-1} with methanol.

4 Apparatus

TSQ 7000 mass spectrometer (Finnigan MAT) with atmospheric pressure ionization (API) interface (ESI mode) or equivalent

High-performance liquid chromatograph with degasser and column oven
HPLC autosampler
HPLC column, Merck, LiChrospher 60 RP Select B, 5 μm, 150 × 4-mm i.d.
Autosampler vials, 1.8-mL
Assorted laboratory glassware
Autopipettes, calibrated

5 Sampling and preparation

No specific sample processing or preparation is needed for this method. Water samples should be warmed to room temperature prior to sampling.

6 Procedure

6.1 Sample setup

Prepare a set of mixed native standard solutions containing concentrations of 0.00, 0.25, 0.50, 2.50, 5.00, 12.50 and 25.00 ng mL^{-1}. Each of these solutions should also contain the mixed internal standards at a concentration of 10 ng mL^{-1}.

Prepare a calibration curve in the following manner. Measure 1 mL of control water into a series of 1.8-mL autosampler vials. Fortify each water sample with 200 μL of the appropriate calibration standard to make a 0.1, 0.5 and 2.5 ng mL^{-1} calibration curve. Mix the sample by vortexing or shaking the vial and analyze 200 μL by LC/ESI-MS/MS. For well and lysimeter water, mix 1 mL of sample water with 200 μL of the 10 ng mL^{-1} mixed internal standard solution in a 1.8-mL autosampler vial, cap the vial, mix the contents and analyze 200 μL by LC/ESIMS/MS.

Table 1 indicates the respective mixed native fortification solution to be added to 1 mL of groundwater to result in the indicated sample fortification level for the respective application and a final volume of 1.2 mL.

6.2 Determination

6.2.1 HPLC conditions

Column	125 × 2-mm i.d. Luna 3u, C_8 (Phenomenex)
Column temperature	40 °C
Flow rate	200 μL min^{-1}
Injection volume	200 μL (200-μL loop)
Split ratio	1 : 1 (post-column, using 0.025-in i.d. PEEK tubing; Upchurch)
HPLC program	Solvent A: water containing 0.1% (v/v) formic acid Solvent B: acetonitrile containing 0.1% (v/v) formic acid A gradient is run from 90% A to 10% A over 12 min. The solvent composition is taken back to 90% A at 12.5 min and equilibrated until 17.5 min (solvents degassed)

Table 1 Preparation of fortification standards for the calibration curves and samples

Sample fortification level (IS = 1 ppb)[a] (ng mL^{-1})	Mixed standard solution		Fortification volume (μL)	Sample use
	Concentration of native solution (ng mL^{-1})	Concentration of internal solution (ng mL^{-1})		
0.0	0	10	200	Linear response, controls, reagent blanks, study samples
0.05	0.25	10	200	Linear response
0.1	0.50	10	200	Calibration curve, linear response, fortified controls
0.5	2.5	10	200	Calibration curve, linear response
1.0	5.0	10	200	Fortified controls
2.5	12.5	10	200	Calibration curve, linear response
5.0	25	10	200	Linear response

[a] The internal standard (IS) is 50% isotopically pure.

6.2.2 Tandem mass spectrometry (MS/MS) conditions

Capillary temperature	275 °C
Sheath gas pressure	100 psi (nitrogen)
Auxiliary pressure	10 mL min^{-1}
SRM scan	See Table 2

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7 Evaluation

7.1 Method

Quantification is performed by comparing the sample response with an average response factor determined from the standard analyses. Internal standards are used

Table 2 SRM scan table and HPLC retention times

Analyte	MW	Precursor ion ($m/z \pm 0.1$)	Product ion ($m/z \pm 0.1$)	Dwell time (s)	Collision offset voltage (V)	Retention time (approx.) (min : s)
Imidacloprid	255	256	209	0.5	−17	6 : 35
[D_3,$^{13}C_1$]Imidacloprid	259	260	213	0.5	−17	6 : 35
Guanidine	210	211	126	0.3	−24	4 : 49
[D_3,$^{13}C_1$]Guanidine	214	215	130	0.3	−24	4 : 49
Urea	211	212	128	0.5	−23	5 : 56
[D_3,$^{13}C_1$]Urea	215	216	132	0.5	−23	5 : 56
Olefinic guanidine	208	209	126	0.3	−26	4 : 47
[D_3,$^{13}C_1$]Olefinic guanidine	212	213	130	0.3	−26	4 : 47

to compensate for signal suppression in electrospray caused by salts or other ionic species present in the water samples.

7.2 Recoveries, limit of detection (LOD) and limit of quantitation (LOQ)

The LODs for imidacloprid and metabolites, when determined by US Environmental Protection Agency (EPA) guidelines,[1] are 0.021–0.024 ng L^{-1}. The LOQ is defined as 0.1 ng L^{-1} for each metabolite. Recoveries from control water samples fortified at the 0.1 and 1 ng L^{-1} levels ranged between 84 and 107% for all metabolites. No relative standard deviation between the respective recoveries for any of the analytes was greater than 10%.

7.3 Calculations

Quantification is based on the use of a three-point calibration curve analyzed in triplicate using ISs to adjust for instrument response. The average response factor from the calibration curve is used for all subsequent analyses.

Calculate the response factor (*RF*) for a calibration standard in solvent using the equation

$$RF = \frac{(\text{Area}_{\text{NAT}})(\text{Conc}_{\text{IS}})}{(\text{Area}_{\text{IS}})(\text{Conc}_{\text{NAT}})}$$

where

Area_{NAT} = the area of response for the product ion from the native standard
Area_{IS} = the area of response for the product ion from the internal standard
Conc_{NAT} = concentration of the native standard (μg L^{-1})
Conc_{IS} = concentration of the internal standard (μg L^{-1})

Perform quantifications using the average response from a three point calibration curve analyzed in triplicate:

$$RF_{\text{AVG}} = \frac{\sum_{i=1}^{9} RF_i}{9}$$

Use the following equation to perform sample calculations:

$$\text{Calc. amount} = \frac{(\text{Area}_{\text{NAT}})(\text{Conc}_{\text{IS}})}{(RF_{\text{AVG}})(\text{Area}_{\text{IS}})}$$

where

Calc. amount = calculated amount (μg L^{-1}), uploaded from the mass spectrometer
Area_{NAT} = the area of response for the native product ion, from the extract
Area_{IS} = the area of response for the internal standard production, from the extract
Conc_{IS} = concentration of the internal standard (μg L^{-1})
RF_{AVG} = average response factor

8 Important points

Although the method is simple and straightforward, there are a few important points to consider. First, since 200 μL are being injected on to a 2-mm diameter HPLC column, compatible solvents must be injected on to the column and the amount of acetonitrile used in the prepared samples should be exactly as directed. Second, the temperature of the column and the use of a degassing system for the mobile phase are critical components required to guarantee reproducible chromatography. In addition, the standards should be stored in a refrigerator when not in use.

Reference

1. 'Definition and Procedure for the Determination of the Method Detection Limit – Revision 1.11', 40 CFR Part 136 Appendix B; *Federal Register,* **49**, 198.

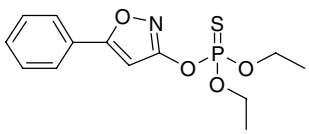
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DropBooks

Isoxathion

Materials to be analyzed	Rice, wheat, fruit, vegetable, potato, sugar cane and tea
Instrumentation	Gas-chromatographic determination for plant materials

1 Introduction

Chemical name (IUPAC)	*O,O*-Diethyl-*O*-5-phenylisoxazol-3-yl phosphorothioate
Structural formula	
Empirical formula	$C_{13}H_{16}NO_4PS$
Molar mass	313.3
Form	Pale yellow liquid
Boiling point	160 °C/0.15 mmHg
Vapor pressure	$<$0.133 mPa at (25 °C)
Log P	3.88 (pH 6.3)
Solubility	Water 1.9 mg L^{-1} (25 °C) Readily soluble in organic solvents
Stability	Unstable in alkaline solutions
Use pattern	Isoxathion is used as an insecticide for rice, orange, tea, various vegetables, soybean, etc.

2 Outline of method

Isoxathion is extracted from plant materials with aqueous acetone. The extracts are concentrated and partitioned with n-hexane after addition of sodium chloride. The n-hexane phase is collected and concentrated after dehydration. The extract is partitioned with n-hexane and acetonitrile. The acetonitrile phase is collected, concentrated, and subjected to Florisil column chromatography. Isoxathion is eluted with diethyl ether–n-hexane after washing the column with the solvent. Isoxathion in the eluate is concentrated and dissolved in acetone and injected into a gas chromatograph for quantitative determination.

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3 Apparatus

Gas chromatograph: HP-6890 equipped with a nitrogen–phosphorus detector (Hewlett-Packard)
Integrator: HP-3396C integrator (Hewlett-Packard)
Rotary evaporator: Model NE-1 (Tokyo Rikakikai Co., Japan)
Erlenmeyer flask, 500-mL
Round bottom flask, 500-mL
Separatory funnel, 500-mL
Glass chromatography column

4 Reagents

Isoxathion: analytical standard, >98% purity
Acetone, acetonitrile, n-hexane, diethyl ether, sodium chloride, anhydrous sodium sulfate: reagent grade (Wako Pure Chemical Inc., Japan)
Florisil for column chromatography, 60–100 mesh (Floridin Co.)
Diatomaceous earth for chemical analysis

5 Analytical procedure

5.1 Extraction

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A 50-g amount (in the case of powder tea, 25 g) of each minced and homogenized plant sample is weighed into a 500-mL flask with a ground stopper and 100 mL of water are added. After standing for 2 h, 150 mL of acetone are added and the flask is vigorously shaken with a shaker for 30 min. The mixture is filtered by suction through a filter paper with a layer of diatomaceous earth 1-cm deep. The residue on the filter paper is returned to the flask and re-extracted with 100 mL of acetone by shaking for 10 min and the mixture is filtered. The combined filtrate in the round-bottom flask is concentrated to less than 100 mL under reduced pressure below 40 °C.

5.2 Partition of n-hexane and aqueous solution

To the concentrated solution, 200 mL of 5% sodium chloride aqueous solution and 100 ml of n-hexane are added and vigorously shaken in a separatory funnel for 5 min. After leaving for a while, the n-hexane layer is collected. To the aqueous layer 100 mL of n-hexane are added and the partition procedure is repeated. The combined n-hexane layer is dried by passing through a funnel containing 50 g of anhydrous sodium sulfate and is concentrated under reduced pressure below 40 °C.

5.3 Partition of acetonitrile and hexane

To the concentrated sample, 30 mL of acetonitrile and 30 mL of n-hexane are added and shaken for 5 min. The acetonitrile layer is collected. To the n-hexane layer, 30 mL of acetonitrile are added and shaken for 5 min and the acetonitrile layer is collected.

The acetonitrile extracts are combined and concentrated under reduced pressure below 40 °C.

5.4 Florisil column chromatography

A glass chromatography column (1.5-cm i.d., 30-cm length) is filled with 10 g of Florisil using a solution of diethyl ether–n-hexane (3 : 17, v/v) and 5 g of anhydrous sodium sulfate are placed on the top of the Florisil. The residual sample obtained in Section 5.3 is dissolved in 10 mL of diethyl ether–n-hexane (3 : 17, v/v) and transferred on to the column and 100 mL of diethyl ether–n-hexane (3 : 17, v/v) are added as eluent and discarded. Using 100 mL of diethyl ether–n-hexane (3 : 7, v/v), isoxathion is eluted. The eluate is collected and concentrated under reduced pressure below 40 °C.

5.5 Determination

The concentrated sample is dissolved in 2 mL of acetone and 2 μL of the solution are injected into a previously conditioned gas chromatograph and the residue concentration is determined.

Operating conditions

Gas chromatograph	Hewlett-Packard Model 6890 equipped with a nitrogen–phosphorus flame ionization detector
Capillary column	Capillary column for gas–liquid chromatography (GLC), DB-1, 0.53-mm i.d. × 15 m, 1-μm film thickness (J&W Scientific)
Column temperature	150 °C, held for 1 min, increased at 10 °C min^{-1} to 240 °C, held for 5 min
Injection port temperature	200 °C
Detector temperature	250 °C
Gas flow rates	Helium carrier gas, 4.2 mL min^{-1} Hydrogen, 3 mL min^{-1} Air, 60 mL min^{-1}
Injection volume	2 μL
Retention time	11 min

6 Evaluation

6.1 Method

Quantitation is performed by the calibration technique. A fresh calibration curve is constructed with isoxathion standard solutions. The calibration curve is plotted as the peak height against the amount of isoxathion injected.

6.2 Limit of detection

The limit of detection of isoxathion in vegetables by this method is 0.004 mg kg^{-1}, as shown below.
Minimum detectable amount: 0.2 ng
Detection limit = (0.2 ng × 2 mL)/(2 μL × 50 g) = 0.004 mg kg^{-1}
Sample volume injected: 2 μL
Final solution volume: 2 mL
Sample weight: 50 g

6.3 Recovery rate in plants

The recovery of isoxathion from vegetables fortified at the 0.1 mg kg^{-1} level by this method is more than 94%.

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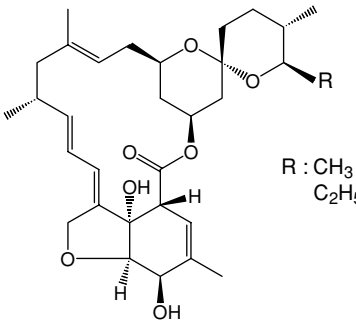
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Milbemectin

Materials to be analyzed	Cucumber, egg plant, strawberry, apple, pear, melon, watermelon, peach, mandarin orange, summer orange and soil
Instrumentation	High-performance liquid chromatographic determination for plant materials and soils

1 Introduction

Milbemectin consists of two active ingredients, $M.A_3$ and $M.A_4$ ($M.A_3/M.A_4 = 30:70$)

Chemical name (IUPAC)	$M.A_3$: (10*E*, 14*E*, 16*E*, 22*Z*)-(1*R*, 4*S*, 5′*S*, 6*R*, 6′*R*,8*R*,13*R*, 20*R*, 21*R*, 24*S*)-21, 24-Dihydroxy-5′, 6′ ,11, 13,22-pentamethyl-3,7,19-trioxatetracyclo[15.6.1.$1^{4,8}$.$0^{20,24}$] pentacosa-10,14,16,22-tetraene-6-spiro-2′-tetrahydropyran-2-one $M.A_4$: (10*E*, 14*E*, 16*E*, 22*Z*)-(1*R*, 4*S*, 5′*S*, 6*R*, 6′*R*,8*R*,13*R*, 20*R*, 21*R*, 24*S*)-6′-Ethyl-21, 24-dihydroxy-5′, 11, 13, 22-tetramethyl-3, 7, 19-trioxatetracyclo [15.6.1.$1^{4,8}$.$0^{20,24}$] pentacosa-10,14,16,22-tetraene-6-spiro-2′-tetrahydropyran-2-one
Structural formula	 R : CH_3 = $M.A_3$ C_2H_5 = $M.A_4$
Empirical formula	$M.A_3$: $C_{31}H_{44}O_7$ $M.A_4$: $C_{32}H_{46}O_7$
Molar mass	$M.A_3$: 528.7 $M.A_4$: 542.7
Form	White crystalline powder

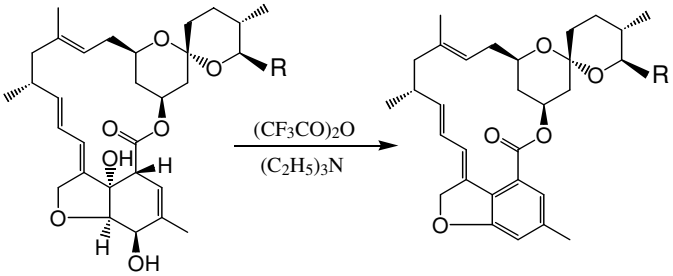
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Melting point	$M.A_3$: 212–215 °C $M.A_4$: 212–215 °C
Vapor pressure	$M.A_3$: $<1.3 \times 10^{-5}$ mPa (20 °C) $M.A_4$: $<1.3 \times 10^{-5}$ mPa (20 °C)
Solubility	Water $M.A_3$ 0.88, $M.A_4$ 7.2 mg L^{-1} (20 °C) Readily soluble in organic solvents
Use pattern	Milbemectin is an acaricide for tea, orange, apple, pear, peach, grape, strawberry, apple, pear, eggplant, watermelon, cucumber, etc.
Regulatory position	The definition of residue is for the parent, milbemectin only

2 Outline of method

Milbemectin consists of two active ingredients, $M.A_3$ and $M.A_4$. Milbemectin is extracted from plant materials and soils with methanol–water (7 : 3, v/v). After centrifugation, the extracts obtained are diluted to volume with the extraction solvent in a volumetric flask. Aliquots of the extracts are transferred on to a previously conditioned C_{18} solid-phase extraction (SPE) column. Milbemectin is eluted with methanol after washing the column with aqueous methanol. The eluate is evaporated to dryness and the residual milbemectin is converted to fluorescent anhydride derivatives after treatment with trifluoroacetic anhydride in 0.5 M triethylamine in benzene solution. The anhydride derivatives of $M.A_3$ and $M.A_4$ possess fluorescent sensitivity. The derivatized samples are dissolved in methanol and injected into a high-performance liquid chromatography (HPLC) system equipped with a fluorescence detector for quantitative determination.

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3 Apparatus

Excel-auto homogenizer: Model EX-10 equipped with a 250-mL centrifuge tube (Nihon Seiki Seisakusyo Co., Japan)
Volumetric flask, 200-mL
Glass filter (17G-3)
Round-bottom flask, 500-mL
Incubator: Model EL-11 (Tokyo Rikakikai Co., Japan)

SPE column: Bakerbond SPE C_{18}, 6-mL (J. T. Baker Inc., USA)
High-speed centrifuge: Model CM-60 (Tomy Seiko Co., Japan)
Rotary evaporator: Model NE-1 (Tokyo Rikakikai Co., Japan)
High-performance liquid chromatograph: Model LC-6A equipped with RF-535 fluorescence detector (Shimadzu Co., Japan)
Data calculator: Chromatopac Model C-R3A (Shimadzu Co., Japan)

4 Reagents

$M.A_3$: analytical standard, >99% purity
$M.A_4$: analytical standard, >99% purity
Methanol, ethanol, acetonitrile, benzene, trifluoroacetic anhydride, triethylamine, distilled water: reagent grade (Wako Pure Chemical Industries, Ltd, Japan)
Methanol, distilled water: specially prepared reagent for HPLC (Wako Pure Chemical Industries, Ltd, Japan)

5 Sample preparation

The plant samples are prepared prior to analysis as follows.

Apple and pear: Sample is minced and homogenized after removing the core and parts around hollows in the top and bottom of the fruit.
Eggplant and strawberry: Sample is minced and homogenized after removing calyxes.
Watermelon and melon: Sample is minced and homogenized after removing pericarp.
Cucumber: Sample is minced and homogenized after removing peduncle.
Peach: Sample is divided into pulp and peel (exocarp), and these two parts are minced and homogenized.

6 Procedure

6.1 Extraction

6.1.1 Plant materials

A 50-g homogenized plant sample is weighed into a centrifuge tube and blended at 7000 rpm with 100 mL of methanol–water (7 : 3, v/v) for 10 min. The resulting mixture is centrifuged at 5000 rpm for 5 min. The supernatant is collected in a 200-mL volumetric flask with suction. In the case of strawberry and peach, the supernatant is filtered through a glass filter (17G-3) previously packed with 10 g of Celite 545. The residue is re-extracted with 50 mL of the same aqueous methanol in the same manner as described above, and the supernatant is collected in a 200-mL volumetric flask. The volumetric flask is filled to the mark with the same aqueous methanol.

6.1.2 Soil

A 50-g (based on the oven-dry weight) soil sample is weighed into a centrifuge tube and blended at 7000 rpm with 100 mL of methanol for 10 min. The resulting

mixture is centrifuged at 5000 rpm for 5 min. The supernatant is collected in a 500-mL round-bottom flask through a glass funnel containing a plug of glass wool. This extraction procedure is repeated three times, and the residue on the glass funnel is rinsed with methanol. The combined extract is concentrated below 45 °C under reduced pressure. The residue is dissolved in methanol–water (7 : 3, v/v) and transferred into a 200-mL volumetric flask, and the flask is filled to volume with the same aqueous methanol solution.

6.2 Cleanup

A 20-mL aliquot of the aqueous methanol extract obtained from above (corresponding to 5 g of each sample) is transferred on to the column previously conditioned by washing successively with 10 mL of methanol and 10 mL of distilled water, and the sample solution is drained. The column is washed with three portions of 5 mL of methanol–water (7 : 3, v/v), and then $M.A_3$ and $M.A_4$ are eluted from the column with 3 mL of methanol. To the eluate, 10 mL of methanol are added and the mixture is concentrated below 45 °C under reduced pressure.

6.3 Conversion of $M.A_3$ and $M.A_4$ to corresponding fluorescent anhydride derivatives

To the residual $M.A_3$ and $M.A_4$ sample obtained from above are added 1 mL of 0.5 M triethylamine in benzene solution and 0.1 mL of trifluoroacetic anhydride, and the reaction mixture is shaken under tightly sealed conditions at 40 °C for 30 min. After the derivatization reaction, 0.05 mL of triethylamine is added to each reaction mixture, and the reaction mixture is concentrated with a rotary evaporator below 45 °C.

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6.4 Determination by HPLC

The anhydride derivatives obtained as above are dissolved in an appropriate volume of methanol, and a 10-μL aliquot of each solution is injected into the pre-conditioned HPLC system. The peak heights of the fluorescent derivatives of $M.A_3$ and $M.A_4$ are converted to weight using a calibration curve corresponding to each chemical.

Operating conditions

HPLC system	Model LC-6A equipped with an RF-535 fluorescence detector (Shimadzu Co., Japan)
Column	Cosmosil $5C_{18}$, 4.6-mm i.d. × 150-mm length (Nakalai Tesque, Japan)
Column temperature	40 °C
Mobile phase	Methanol–water (49 : 1, v/v)
Flow rate	1 mL min^{-1}
Detector	Fluorescent detector: Excitation 360 nm, emission 460 nm
Injection volume	10 μL
Retention time	Fluorescent derivative of $M.A_3$ 6 min and of $M.A_4$ 7 min

7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. A standard solution containing 0.1 mg kg^{-1} of both M.A$_3$ and M.A$_4$ is prepared and 1, 2.5, 5 and 7.5 mL of this solution are pipetted into a round-bottom flask separately and evaporated. Each sample is converted into the fluorescent anhydride derivative according to the procedures described above. Each sample is dissolved in 10 mL of methanol for injection into the HPLC system. The calibration curves are obtained by plotting the peak heights against the amounts of M.A$_3$ and M.A$_4$. The derivatives for preparing the calibration curve should be freshly prepared on a daily basis prior to quantitation.

7.2 Limit of detection

The limit of detection for this method is 0.02 mg kg^{-1} for each chemical, as shown, below.

Minimum detectable amount: 0.1 ng (equivalent to M.A$_3$ or M.A$_4$)
Sample volume injected: 10 μL
Final solution volume: 10 mL
Sample weight: 5 g
Detection limit = (0.1 ng × 10 mL)/(10 μL × 5 g) = 0.02 mg kg^{-1}

7.3 Recovery from plants

The recoveries of M.A$_3$ and M.A$_4$ from plant materials fortified at the 0.1 mg kg^{-1} level ranged from 77 to 98%.

7.4 Recovery from soil

The recoveries of M.A$_3$ and M.A$_4$ from soils fortified at the 0.2 mg kg^{-1} level ranged from 79 to 85%.

8 Important points

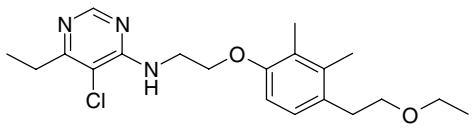
The fluorescent derivatives of M.A$_3$ and M.A$_4$ gradually decrease in fluorescence intensity at room temperature. The derivatives intended for both calibration curve and residual samples should be quickly quantified by HPLC after preparation.

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Pyrimidifen

Materials to be analyzed	Apple, pear, orange, peach, strawberry, cabbage, radish, tea and soil
Instrumentation	High-performance liquid chromatographic determination for plant materials

1 Introduction

Chemical name (IUPAC)	5-Chloro-*N*-{2-[4-(2-ethoxyethyl)-2,3-dimethylphenoxy] ethyl}-6-ethylpyrimidin-4-amine
Structural formula	
Empirical formula	$C_{20}H_{28}ClN_3O_2$
Molar mass	377.9
Form	Colorless crystals
Melting point	69.4–70.9 °C
Vapor pressure	1.6×10^{-4} mPa (25 °C)
Log P	4.59 (23 °C)
Solubility	Water 2.17 mg L^{-1} (25 °C)
Stability	Stable in acid and alkali
Use pattern	Pyrimidifen is used as an acaricide for apple, pear, orange, peach, strawberry, cabbage, radish and tea
Regulatory position	The definition of residue is for the parent, pyrimidifen, only

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2 Outline of method

Pyrimidifen is extracted from plant materials with methanol–water (7 : 3, v/v). The extracts are concentrated and pyrimidifen is partitioned with n-hexane after addition of sodium chloride. The organic phase is collected and concentrated. Pyrimidifen in the organic phase is purified by silica gel column chromatography. Pyrimidifen is dissolved in acetonitrile and injected into a high-performance liquid chromatography

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(HPLC) system equipped with an ODS column and ultraviolet (UV) detector for quantitative determination.

3 Apparatus

Volumetric flask, 300-mL
Round-bottom flask, 500-mL
Separatory funnel, 150-mL
Rotary evaporator: Model NE-1 (Tokyo Rikakikai Co., Japan)
High-performance liquid chromatograph: Model LC-10A (Shimadzu Co., Japan)
Integrator: Chromatopac Model C-R7A (Shimadzu Co., Japan)

4 Reagents

Pyrimidifen: analytical standard, >99% purity
Methanol, n-hexane, ethyl acetate, distilled water, sodium chloride, sodium sulfate, reagent grade for residue analysis (Wako Pure Chemical Industries, Ltd, Japan)
Acetonitrile, methanol, distilled water, reagent grade for high-performance liquid chromatography
Silica gel: for column chromatography (200-mesh, Nakalai Tesque, Japan)
Celite No. 545: for chemical analysis (Nakalai Tesque, Japan)

5 Sampling and preparation

No specific sample preparation and processing are needed for this method.

6 Procedure

6.1 Extraction

A 10-g amount of each minced and homogenized plant sample is weighed into a 300-mL volumetric flask, 100 mL of methanol–water (7 : 3, v/v) are added and the mixture is shaken vigorously for 30 min. The resulting mixture is filtered with a glass filter by suction. The residue is re-extracted with 100 mL of the same solvent and filtered. The combined aqueous methanol extracts are transferred into a 500-mL round-bottom flask and concentrated to 30 mL under reduced pressure below 45 °C.

6.2 Cleanup

6.2.1 Partition into n-hexane

The resulting solution obtained from above is transferred into a 150-mL separatory funnel and 50 mL of n-hexane and 3 g of sodium chloride are added. After shaking

for 5 min, the n-hexane layer is collected. To the aqueous layer, 50 mL of n-hexane are added and the partition procedure is repeated. The combined n-hexane extracts are dried by passing through a funnel containing 50 g of anhydrous sodium sulfate. The filtrate is collected in a round-bottom flask and concentrated to dryness under reduced pressure below 45 °C.

6.2.2 Silica gel column chromatography

A 5-g amount of a mixture of silica gel and Celite 545 (3 : 1, w/w) is packed into a glass column (2-cm i.d., length 30-cm) with n-hexane–ethyl acetate (4 : 1, v/v). The residue obtained from Section 6.2.1 is dissolved in 15 mL of n-hexane–ethyl acetate (4 : 1, v/v), transferred on to the column and drained. The column is washed with another 35 mL of the same solvent and drained. Pyrimidifen is eluted with 150 mL of the same solvent. The eluate is concentrated to dryness under reduced pressure below 45 °C.

6.3 Determination

The cleaned-up sample is dissolved in 4 mL of acetonitrile and 20 μL of the solution are injected into the previously conditioned HPLC system and the residue concentration is determined.

Operating conditions

Column	Cosmosil $5C_{18}$ 4.6-mm i.d. × 250-mm length (Nakalai Tesque, Japan)
Mobile phase	Methanol–acetonitrile–water (9 : 6 : 5, v/v/v)
Flow rate	1 mL min^{-1}
Column temperature	40 °C
UV wavelength	245 nm
Retention time	17 min

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7 Evaluation

7.1 Method

Quantitation is performed by the calibration technique. The calibration curve is constructed with pyrimidifen standard solutions, plotting the peak height against the injected amount of pyrimidifen.

7.2 Limit of detection

The limit of detection of pyrimidifen by this method is 0.005 mg kg^{-1}, as shown below.

Minimum detectable amount: 0.25 ng
Detection limit = (0.25 ng × 4 mL)/(20 μL × 10 g) = 0.005 mg kg^{-1}

Sample volume injected: 20 μL
Final solution volume: 4 mL
Sample weight: 10 g

7.3 Recovery

The recovery of pyrimidifen from an apple fruit sample fortified at the 0.2 mg kg^{-1} level is 95%.

7.4 Calculation of residue

The residue R, expressed in mg kg^{-1} pyrimidifen, is calculated using the following equation:

$$R = (W \times V_{End})/(V_i \times G)$$

where

G = sample weight (g)
V_{End} = terminal volume of sample solution from Section 6.3 (mL)
V_i = portion of volume of V_{End} injected into the HPLC system (μL)
W = amount of pyrimidifen for V_i read from the calibration curve (ng)

8 Important point

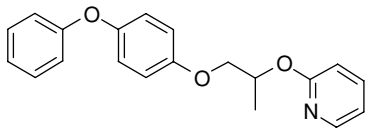
Pyrimidifen residues in fruits such as apple and orange are stable (>90%) after frozen storage for 2 months.

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Pyriproxyfen

Materials to be analyzed	Cabbage, cantaloupe, cauliflower, citrus fruit, cottonseed, cucumber, mustard greens, nutmeats, pome fruit, stone fruit, summer squash, tomatoes, soil, and water
Instrumentation	Gas chromatograph with nitrogen–phosphorus detector

1 Introduction

Chemical name (IUPAC)	4-Phenoxyphenyl (*RS*)-2-(2-pyridyloxy)propyl ether
Structural formula	
Empirical formula	$C_{20}H_{19}NO_3$
Molecular mass	321.4
Melting point	47 °C
Vapor pressure	$< 1 \times 10^{-7}$ mmHg at 22.8 °C
Solubility	Water: 0.37 mg L^{-1} at 20 °C Hexane: 80 g L^{-1} at 20 °C Methanol: 60 g L^{-1} at 20 °C
Stability	Stable in acidic, neutral and basic aqueous solutions
Other properties	Pale yellowish solid, faint characteristic odor Flash point: 119 °C (Pensky–Martens closed tester)
Use pattern	Pyriproxyfen is an insect growth regulator which acts both as an ovacide and as an inhibitor of development (juvenile hormone mimic) against white flies, scale, and psylla. The specificity of pyriproxyfen, and its low mammalian toxicity, allow for some variation in application timing. For example, the lack of toxicity to bees allows pyriproxyfen to be applied during bloom on apple trees, and its low mammalian toxicity allows for a very short pre-harvest interval on citrus
Regulatory position	The residue definition is for pyriproxyfen alone

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2 Outline of method

2.1 Fruits and vegetables

Residues are extracted with acetone. The extract is rotary evaporated to remove acetone, the concentrated residue is diluted with 5% aqueous sodium chloride, and residues are partitioned into dichloromethane. The extract is then concentrated and purified on a silica gel column. Residues of pyriproxyfen are quantitated by gas chromatography with nitrogen–phosphorus detection (GC/NPD). For citrus, a hexane–acetonitrile solvent partition step is required for oil removal prior to the dichloromethane partition step.

2.2 Ginned cottonseed

Residues are extracted with acetonitrile–water (4 : 1, v/v). The extract is rotary evaporated to remove acetonitrile, and the concentrated residue is diluted with 5% aqueous sodium chloride prior to partitioning with dichloromethane. The dichloromethane is removed by rotary evaporation and the sample extract is purified by partitioning between hexane–acetonitrile and by silica gel chromatography. Residues of pyriproxyfen are quantitated by GC/NPD.

2.3 Nutmeats

Residues are extracted with acetone. The extract is then rotary evaporated to remove acetone, the concentrated residue is diluted with 5% aqueous sodium chloride, and the residues are partitioned into dichloromethane. The dichloromethane is removed by rotary evaporation, and the sample extract is purified by partitioning between hexane–acetonitrile and by silica gel chromatography. A second hexane–acetonitrile partitioning step is required to remove residual oil, and the residues of pyriproxyfen are quantitated by GC/NPD.

2.4 Soil

Residues are extracted with methanol–0.1 M sodium hydroxide (NaOH) (4 : 1, v/v). The extract is rotary evaporated to remove the methanol, the concentrated residue is diluted with water (at neutral pH), and the residues are partitioned into dichloromethane. The extract is purified using an alumina column. Pyriproxyfen residues are quantitated by GC/NPD.

2.5 Water

Residues are partitioned into ethyl acetate. The extract is purified using a Florisil column, and pyriproxyfen residues are quantitated by GC/NPD.

3 Apparatus

Buchner funnel, 9-cm diameter
Centrifuge tubes, 15-mL
Centrifuge
Filter paper, 9-cm diameter Whatman GF/A
Filter flask, 500- and 1000-mL
Filter funnel, 10-cm diameter
Gas chromatograph, equipped with a nitrogen–phosphorus detector
Glass chromatography column, 19 × 300 mm with Teflon stopcock
Glass wool (Pyrex)
Linear shaker
OmniMixer, with an adapter for a pint Mason jar
Pasteur pipets
pH indicator paper
Rotary vacuum evaporator, with a 40 °C water-bath
Round-bottom flasks, 50-, 100-, 500- and 1000-mL
Separatory funnels, 250- and 500-mL
Ultrasonic water-bath
Vortex mixer

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4 Reagents

Acetone, reagent grade
Acetonitrile, reagent grade
Deionized water
Dichloromethane, reagent grade
Diethyl ether, reagent grade
Ethyl acetate, reagent grade
Hexane, reagent grade
Methanol
Phosphoric acid, 85%, reagent grade
Phosphoric acid, 1 M solution
Toluene, reagent grade
Alumina, 150 mesh (Aldrich, Catalog No. 19,996-6)
Florisil, 60–100 mesh
Silica gel, 70–230 mesh (EM Science, Catalog No. 7734-7)
Sodium chloride, reagent grade
Sodium chloride solution, 5% aqueous solution
Sodium hydroxide, reagent grade
Sodium hydroxide solution, 0.1 M aqueous solution
Sodium sulfate, anhydrous

5 Sampling and preparation

No specific sample preparation or processing is needed for this method. In general, fruits and vegetables were macerated with dry-ice and placed into freezer storage prior to extraction.

6 Procedure

6.1 Extraction and cleanup

6.1.1 Fruit and vegetables

Extraction. Homogenize 20 g of sample in a pint Mason jar with 150 mL of acetone for approximately 5 min using an OmniMixer. Allow the solids to settle and decant the liquid through a Whatman GF/A glass-fiber filter in a Buchner funnel, collecting the filtrate in a 500-mL (or 1000-mL) filter flask. Repeat the extraction and filtration twice with additional 150-mL portions of acetone. Following the third extraction, transfer the solids onto the filter. Rinse the extraction jar with approximately 20 mL of acetone and add this rinse to the solids on the Buchner funnel, combining the rinse with the other extracts. Transfer the filtrate to a 1000-mL round-bottom flask, and remove the acetone by rotary evaporation under reduced pressure in a $<40\,^{\circ}$C water-bath. For citrus samples only, add 250 mL of ethyl acetate to reduce the water volume to <5 mL. For the non-oily crops, the volume of water remaining is not critical. For all samples except citrus, proceed to the dichloromethane partition step. For citrus, proceed to the hexane–acetonitrile partition step.

Hexane–acetonitrile partition. Add 70 mL of acetonitrile (hexane-saturated) to the round-bottom flask and briefly sonicate the mixture to dislodge the material on the glass surface. Transfer the mixture into a 250-mL separatory funnel, add 100 mL of hexane (acetonitrile-saturated), and shake the funnel vigorously for 1 min. Allow the phases to separate, then drain the acetonitrile layer into a 500-mL round-bottom flask. Repeat the partitioning of the hexane layer twice with 70-mL portions of acetonitrile (hexane-saturated), combining the three extracts in the 500-mL round-bottom flask. Evaporate the acetonitrile (just to dryness) by rotary evaporation under reduced pressure with a $<40\,^{\circ}$C water-bath. Proceed to the dichloromethane partition step.

Dichloromethane partition. Add 100 mL of 5% sodium chloride solution to the round-bottom flask, briefly sonicate the flask to dislodge the residues, and transfer the mixture into a 500-mL separatory funnel. Rinse the round-bottom flask with 150 mL of dichloromethane and transfer the dichloromethane to the separatory funnel. Shake the funnel vigorously (with occasional venting) for 1 min and allow the phases to separate. Drain the dichloromethane through sodium sulfate (approximately 50 g suspended on a glass-wool plug in a 10-cm diameter filter funnel) into a 1000-mL round-bottom flask. Once the extract has drained through the sodium sulfate, rinse the sodium sulfate with approximately 20 mL of dichloromethane. Repeat the

partition twice with 100-mL portions of dichloromethane, rinsing as before. Rotary evaporate the combined dichloromethane extract to 20–30 mL under reduced pressure in a <40 °C water-bath. Transfer the residues to a 100-mL round-bottom flask, rinsing the 1000-mL flask twice with 10-mL portions of ethyl acetate. Continue the evaporation, and evaporate the sample just to dryness. Add 5 mL of hexane–ethyl acetate (4 : 1, v/v), stopper the flask, and sonicate the sample to dissolve the residues.

Silica gel column cleanup. Prepare a silica gel column by placing a glass-wool plug in the bottom of a glass chromatography column. Slurry 18 g of silica gel with hexane–ethyl acetate (4 : 1, v/v) and pour the slurry into the column. Rinse the walls of the column with hexane–ethyl acetate, and add approximately 2 g of sodium sulfate to the top of the silica gel column. Drain the solvent to the top of the sodium sulfate layer.

Transfer the sample to the column. Rinse the sample flask sequentially with 5 mL, 5 mL, and then 10 mL of hexane–ethyl acetate (4 : 1, v/v). Allow each rinse to drain to the top of the sodium sulfate layer before adding the next portion. Discard the accumulated eluant, place a 100-mL round-bottom flask under the column, and elute the pyriproxyfen residues with 55 mL of hexane–ethyl acetate (4 : 1, v/v). Evaporate the eluate by rotary evaporation under reduced pressure in a <40 °C water-bath and reconstitute the sample in 2.0 mL of toluene with sonication for analysis (Section 6.2).

6.1.2 Cottonseed

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Extraction. Homogenize 10 g of a prepared sample in a pint Mason jar with 100 mL of acetonitrile–water (4 : 1, v/v) for approximately 5 min using an OmniMixer. Filter the sample through a Whatman GF/A glass-fiber filter in a Buchner funnel, collecting the filtrate in a 500-mL filter flask. Transfer the filter cake back into the jar and repeat the extraction and filtration. Rinse the extraction jar twice with approximately 20 mL of acetonitrile, passing each rinse through the solids on the Buchner funnel and combining the rinses with the other extracts. Transfer the filtrate to a 1000-mL round-bottom flask, add 150 mL of ethyl acetate to inhibit foaming, and rotary evaporate the solvent under reduced pressure in a <40 °C water-bath (approximately 20–40 mL of water will remain).

Dichloromethane partition. Transfer the aqueous extract into a 500-mL separatory funnel and add 150 mL of 5% sodium chloride solution. Rinse the round-bottom flask with 80 mL of dichloromethane and transfer into the separatory funnel. Shake the separatory funnel vigorously (with occasional venting) for 1 min and allow the phases to separate. Drain the lower dichloromethane through sodium sulfate (approximately 50 g suspended on a glass-wool plug in a 10-cm diameter filter funnel, pre-rinsed with 25 mL of dichloromethane) into a 500-mL round-bottom flask. Repeat the partition with another 80-mL portion of dichloromethane. Drain the dichloromethane through the sodium sulfate as before, and rinse the sodium sulfate with three 10-mL portions of dichloromethane. Evaporate the combined dichloromethane extract just to dryness using rotary evaporation under reduced pressure in a <40 °C water-bath.

Hexane–acetonitrile partition. Add 150 mL of hexane (acetonitrile-saturated) to the round-bottom flask to reconstitute the sample, and transfer the sample to a 500-mL separatory funnel. Rinse the round-bottom flask with 100 mL of acetonitrile (hexane-saturated), and add this rinse to the separatory funnel. Shake the funnel vigorously for 1 min, allow the phases to separate and drain the acetonitrile layer into a clean 500-mL round-bottom flask. Repeat the partitioning of the hexane layer with a second 100-mL portion of acetonitrile (hexane saturated), combining the acetonitrile layers. Rotary evaporate the extract under reduced pressure in a $<40\,^{\circ}$C water-bath to approximately 40–50 mL. Transfer the extract to a 100-mL round-bottom flask, rinsing the 500-mL round-bottom flask three times with 5 mL of acetonitrile (hexane-saturated). Continue the evaporation and take the sample just to dryness. Reconstitute the sample by sequentially adding 1 mL of toluene and 2 mL of hexane to the 100-mL round-bottom flask. Sonicate the sample to dissolve any residue adhering to the walls of the flask.

Silica gel column cleanup. Prepare a silica gel column by placing a glass-wool plug in the bottom of a glass chromatography column. Slurry 15 g of silica gel (deactivated with 10% water) with hexane, and transfer the slurry to the column. Rinse the walls of the column with hexane and add 2 g of sodium sulfate to the top of the silica gel column. Drain the hexane to the top of the sodium sulfate layer.

Transfer the sample to the column and drain the solvent to the top of the sodium sulfate layer. Rinse the round-bottom flask three times with 3-mL portions of hexane, adding these rinses sequentially to the column and draining the solvent to the top of the sodium sulfate layer before the next addition. Pass 90 mL of hexane through the column, followed by 50 mL of hexane–diethyl ether (15 : 1, v/v). Add each portion of eluting solvent to the round-bottom flask and sonicate the flask before adding the solution to the column. Discard the accumulated eluate. Place a 250-mL round-bottom flask under the column and elute the pyriproxyfen residues with 50 mL of hexane–diethyl ether (15 : 1, v/v), followed by 20 mL of hexane–acetone (7 : 3, v/v). As before, add each portion of eluting solvent to the round-bottom flask and sonicate the flask before adding the solution to the column. Rotary evaporate the combined eluate under reduced pressure in a $<40\,^{\circ}$C water-bath to 40–50 mL. Transfer the sample to a 100-mL round-bottom flask with three 5-mL acetone rinses, and continue rotary evaporation to take the sample just to dryness. Reconstitute the sample in 1.0 mL of toluene with sonication for analysis (Section 6.2).

6.1.3 Nutmeats

Extraction. Extract 20 g of sample as described for fruit and vegetables. Evaporate the acetone extract to dryness by rotary evaporation under reduced pressure in a $<40\,^{\circ}$C water-bath.

Dichloromethane partition. Partition residues into dichloromethane as described for fruit and vegetables. Evaporate the dichloromethane extract just to dryness by rotary evaporation under reduced pressure in a $<40\,^{\circ}$C water-bath.

Hexane–acetonitrile partition. Partition the sample between hexane and acetonitrile as described for fruit and vegetables to remove citrus oils. Evaporate the

dichloromethane extract just to dryness by rotary evaporation under reduced pressure in a <40 °C water-bath.

Silica gel column cleanup. Clean up the sample with a 15-g silica gel column as described for ginned cottonseed. Evaporate the column eluate just to dryness using rotary evaporation under reduced pressure in a <40 °C water-bath.

Second hexane–acetonitrile partition. Transfer the sample to a 15-mL glass centrifuge tube by rinsing the round-bottom flask with 2 mL of hexane (acetonitrile-saturated) followed by 2 mL of acetonitrile (hexane-saturated). Sonicate each rinse for approximately 15 s before transferring the rinse via a Pasteur pipet to the centrifuge tube. Stopper the centrifuge tube, mix the sample for 30 s using a vortex mixer, and allow the phases to separate (centrifuge for approximately 2 min, if necessary). Carefully withdraw the acetonitrile (the lower layer) with a glass syringe or Pasteur pipet, and transfer the acetonitrile to a 50-mL round-bottom flask. Extract the hexane layer with two additional 2-mL portions of acetonitrile (hexane-saturated), rinsing the 250-mL round-bottom flask with each before adding the solvent to the centrifuge tube. Combine all of the acetonitrile layers in the 50-mL round-bottom flask. Evaporate the acetonitrile extract just to dryness by rotary evaporation under reduced pressure in a <40 °C water-bath. Reconstitute the sample in 2.0 mL of toluene with sonication for analysis (Section 6.2).

6.1.4 Soil

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Extraction. Place 20 g of sample (wet-weight basis) in a pint Mason jar, add 40 mL of methanol–0.1 M NaOH (4 : 1, v/v), cap the jar, and shake it for approximately 15 min using a linear shaker. Filter the sample through a Whatman GF/A glass-fiber filter in a Buchner funnel, collecting the filtrate in a 500-mL filter flask. Rinse the jar with 40 mL of methanol–0.1 M NaOH (4 : 1, v/v) and pass the rinse through the filter cake, combining the rinse with the extract. Transfer the filter cake back into the jar and repeat the extraction with a second 40-mL portion of methanol–0.1 M NaOH (4 : 1, v/v). Filter the sample as before, rinsing the jar again with approximately 20 mL of methanol–0.1 M NaOH (4 : 1, v/v) and passing the rinse through the filter cake. Transfer the combined filtrate to a 500-mL round-bottom flask, rinsing the filter flask twice with 20-mL portions of methanol–0.1 M NaOH (4 : 1, v/v). Reduce the volume of the extract to approximately 20 mL by rotary evaporation under reduced pressure in a <40 °C water-bath.

Dichloromethane partition. Add 100 mL of deionized water to the round-bottom flask. Transfer the sample to a 500-mL separatory funnel and add 1 mL of phosphate buffer and 1 g of sodium sulfate. Adjust the pH to 7 with 1 M phosphoric acid (approximately 0.75 mL), checking that the pH is approximately 7 with pH paper. Add 100 mL of dichloromethane to the separatory funnel, rinsing the round-bottom flask with portions of this before addition to the separatory funnel. Shake the separatory funnel vigorously (with occasional venting) for 1 min, and allow the phases to separate. Drain the dichloromethane through sodium sulfate (approximately 50 g

suspended on a glass-wool plug in a 10-cm diameter filter funnel, pre-rinsed with 25 mL of dichloromethane) into a 500-mL round-bottom flask. Repeat the partition with another 100-mL portion of dichloromethane. Drain the dichloromethane through the sodium sulfate as before, and rinse the sodium sulfate with two 10-mL portions of dichloromethane. Evaporate the dichloromethane extract just to dryness by rotary evaporation under reduced pressure in a <40 °C water-bath. Reconstitute the sample in 3 mL of hexane–ethyl acetate (10 : 1, v/v) with sonication.

Alumina column cleanup. Prepare an alumina column by placing a glass-wool plug in the bottom of a glass chromatography column. Slurry 10 g of alumina with hexane–ethyl acetate (10 : 1, v/v), and pour the slurry into the column. Rinse the walls of the column with hexane–ethyl acetate (10 : 1, v/v), and add approximately 2 g of sodium sulfate to the top of the alumina column. Drain the solvent to the top of the sodium sulfate layer.

Transfer the sample to the column with a Pasteur pipet and drain the solvent to the top of the sodium sulfate layer. Rinse the round-bottom flask three times with 3-mL portions of hexane–ethyl acetate (10 : 1, v/v), adding these rinses sequentially to the column and draining the solvent to the top of the sodium sulfate layer before the next addition. Discard the accumulated eluate and place a 100-mL round-bottom flask under the column. Elute the residues with 28 mL of hexane–ethyl acetate (10 : 1, v/v). Evaporate the column eluate just to dryness by rotary evaporation under reduced pressure in a <40 °C water-bath. Reconstitute the sample in 2.0 mL of toluene for analysis (Section 6.2).

6.1.5 Water

Extraction. Transfer 500 mL of the water sample into a 1000-mL separatory funnel. Add 200 mL of ethyl acetate to the separatory funnel and shake vigorously for 1 min. Allow the phases to separate and drain the aqueous layer into a 600-mL beaker (or suitable container). Filter the ethyl acetate through sodium sulfate (approximately 50–70 g suspended on a glass-wool plug in a 10-cm diameter filter funnel) into a 1000-mL round-bottom flask. Once the extract has drained through the sodium sulfate, rinse the sodium sulfate with 10 mL of ethyl acetate. Repeat the partition twice with 100-mL portions of ethyl acetate, draining each extract through the sodium sulfate and rinsing the sodium sulfate as before. Evaporate the sample extract just to dryness by rotary evaporation under reduced pressure in a <40 °C water-bath. Reconstitute the sample in 10 mL of hexane–ethyl acetate (50 : 1, v/v).

Florisil column cleanup. Prepare a Florisil column by placing a glass-wool plug in the bottom of a glass chromatography column. Slurry 15 g of Florisil with hexane–ethyl acetate (50 : 1, v/v) and transfer the slurry into the column. Rinse the walls of the column with hexane–ethyl acetate (50 : 1, v/v) and add approximately 2 g of sodium sulfate to the top of the Florisil column. Drain the solvent to the top of the sodium sulfate layer.

Transfer the sample to the column and drain the solvent to the top of the sodium sulfate layer. Rinse the round-bottom flask twice with 10-mL portions of hexane–ethyl

acetate (50 : 1, v/v), adding these rinses sequentially to the column and draining the solvent to the top of the sodium sulfate layer before the next addition. Wash the column with an additional 25-mL portion of hexane–ethyl acetate (50 : 1, v/v) and discard the accumulated eluate. Place a 250-mL round-bottom flask under the column. Elute the residues with 75 mL of hexane–ethyl acetate (15 : 1, v/v). Reduce the volume of the eluate to 15–20 mL by rotary evaporation under reduced pressure in a <40 °C water-bath. Transfer the sample into a 50-mL round-bottom flask, rinsing the 250-mL round-bottom flask three times with 5-mL portions of hexane–ethyl acetate (15 : 1, v/v). Evaporate the sample just to dryness. Reconstitute the sample in toluene for analysis (Section 6.2).

6.2 Determination

6.2.1 Plant material, soil, and water

Inject the sample extract into a gas chromatograph within an analytical sequence, with calibration standards bracketing and interspersed within the sequence.

Operating conditions

Gas chromatograph	HP5890, Hewlett-Packard
Sample injector	HP7673, Hewlett Packard
Injection port	Split/splitless (approximately 1 : 1 split ratio), 270 °C
Injection mode	Splitless, purge on at 0.6 min
Column	DB-17 (J&W Scientific), 30 m × 0.53-mm i.d., 1.0-μm film thickness
Column temperature	260 °C, 2 min; 10 °C min^{-1} to 280 °C, 6 min (10 min total)
Detector	Nitrogen–phosphorus detector, 300 °C
Gas flow rates	Helium carrier gas, 30 mL min^{-1}
	Hydrogen, 3.6 mL min^{-1}
	Air, 110 mL min^{-1}
Injection volume	1.0 or 2.0 μL
Retention time	2.2 min

Alternative parameters 1

Injection port	Split/splitless (approximately 3 : 1 split ratio), 250 °C
Injection mode	Splitless, purge on at 0.6 min
Column	DB-17 (J&W Scientific), 30 m × 0.53-mm i.d., 1.5-μm film thickness
Column temperature	265 °C, 2.5 min; 10 °C min^{-1} to 280 °C, 6 min (10 min total)
Detector	Nitrogen–phosphorus detector, 300 °C
Gas flow rates	Helium carrier gas, 10 mL min^{-1}
	Helium makeup gas, 20 mL min^{-1}
	Hydrogen, 3.6 mL min^{-1}
	Air, 110 mL min^{-1}
Injection volume	1.0 μL
Retention time	4.2 min

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Alternative parameters 2

Injection port	Split/splitless (approximately 2 : 1 split ratio), 300 °C
Injection mode	Splitless, purge on at 0.6 min
Column	DB-5 (J&W Scientific), 30 m × 0.53-mm i.d., 1.5-μm film thickness
Column temperature	250 °C, 7 min (isothermal)
Detector	Nitrogen–phosphorus detector, 300 °C
Gas flow rates	Helium carrier gas, 20 mL min^{-1} Helium makeup gas, 10 mL min^{-1} Hydrogen, 3.6 mL min^{-1} Air, 110 mL min^{-1}
Injection volume	1.0 μL
Retention time	3.8 min

7 Evaluation

7.1 Method

Prior to use, the linearity of the gas chromatography system should be verified by analyzing at least four standards of different concentrations. The linearity standards should range in concentration from 0.1 to 2.0 μg mL^{-1}. A response factor for each standard is calculated by dividing the response of each standard by its concentration. The relative standard deviation (RSD) of these response factors should be <10%.

Quantitation is performed using the external standard calibration technique. The concentration of the calibration standard is 1.0 μg mL^{-1}. The calibration standard should be injected prior to injection of the treated samples and again after every second or third injection of treated samples. The analytical sequence should end with a calibration standard. The RSD of the calibration standards should be <10%.

7.2 Recoveries, limit of quantitation, and limit of detection

7.2.1 Plant material

Fortification of untreated plant matrices at 0.02 and 0.1 mg kg^{-1} gave recoveries from 67 to 103%. The limit of detection (LOD) is 0.01 mg kg^{-1} and the limit of quantification (LOQ) is 0.02 mg kg^{-1}.

7.2.2 Soil

Fortification of blank soil at 0.02 and 0.1 mg kg^{-1} gave recoveries from 88 to 95%, with an LOD of 0.01 mg kg^{-1} and an LOQ of 0.02 mg kg^{-1}.

7.2.3 Water

Fortification of blank water between 2.0 and 11 μg L^{-1} gave recoveries from 87 to 107%, with an LOD of 1.0 μg L^{-1} and an LOQ of 2.0 μg L^{-1}.

7.3 Calculation of residues

$$\text{Pyriproxyfen (mg kg}^{-1}\text{ or } \mu\text{g L}^{-1}) = \frac{A \times C \times V}{B \times S}$$

where

A = integration counts for pyriproxyfen in the sample
C = concentration of pyriproxyfen in the calibrating standard (1.0 μg mL^{-1})
V = final volume of the sample extract (mL)
B = mean integration counts for the calibration standards
S = sample weight or volume (g or L)

8 Important points

During evaporation of organic solvents, the temperature of the water-bath should be kept at 40 °C or lower. Once the solvent is evaporated, continued rotary evaporation may lead to reduced analyte recovery.

In general, pyriproxyfen residues are stable in macerated crop samples. Stability problems have been observed in summer squash, and this should be extracted within 21 days of harvest.

The variety of cleanup columns included may allow for rapid adaptation to additional matrices. If using hexane–diethyl ether (15 : 1, v/v) as an eluent, this solution should be prepared just prior to use. Cleanup with silica gel and hexane–ethyl acetate (4 : 1, v/v) is recommended for most crop samples.

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Each batch of alumina, Florisil, and silica gel used in a cleanup column must be checked for recovery of pyriproxyfen. If the recovery of pyriproxyfen is <90%, the elution volume and/or solvent mixture must be adjusted until suitable recoveries are obtained.

Cleanup of highly colored samples (e.g., mustard greens) on silica columns may require that only half of the sample extract be passed through the silica column.

Charles A. Green
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